Basidiospores: their influence on our thinking regarding management of *Ganoderma* root-rot disease in tropical hardwood plantation crops.

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ABSTRACT

Forest products are a major source of national revenue in Indonesia. Nearly 33% (69.9 million ha) of the country's land area includes production forests. Several *Acacia* species are planted mainly for pulp production. By 2014 the total area of hardwood plantations established in this country was reported as approximately 1.2 M hectares with 600,000 ha of *Acacia mangium*. However due to susceptibility to two diseases (*Ganoderma philippii* and *Ceratocystis manginecans*) the *A. mangium* has been replaced by less susceptible eucalypts.

By the early 2000s it was acknowledged that *G. philippii*, a basidiomycete root rot pathogen, was causing serious damage to *A. mangium*. In Central Sumatra, by the third rotation, several sites were found to be no longer capable of providing a commercial yield at harvest. Root diseases caused by basidiomycetes are among the most studied diseases of trees in the world, in particular for the genera *Armillaria* and *Heterobasidion*. Much can be inferred from those studies for *G. phillipii* but very little has been substantiated. The ability to trade-off concepts such as disease risk with the cost, effectiveness and deployment of various management strategies will depend on how well the pathosystem is understood and the context imposed by the particular cropping system and commercial environment. The main objective of the present study was to define fundamental aspects of *G. philippii* disease biology and aetiology (such as sexuality, gene flow and capacity for genetic variation, population dynamics) in order to ascertain the importance of basidiospores in disease dispersal and incidence.

The study comprised three experimental investigations of; (1) *G. philippii* basidiospore germination, (2) *G. philippii* breeding systems, and (3) population structures of *G. philippii* in acacia plantations. Two other saprophytic *Ganoderma* species that are frequently found fruiting in *Acacia* and *Eucalyptus* plantations were included in the first two studies, for comparison with *G. philippii*.

Spores were collected opportunistically and non-destructively from fresh basidiocarps emanating from the stems of mature-aged trees. Microscopic observations and empirical mathematical modelling enabled a description of the germination dynamics of the basidiospores of *G. philippii*, *G. mastoporum* and *G. australe* as influenced by factors such as spore density, simple or complex carbohydrate availability in the nutrient media, biotic carbon sources such as sawdust and/or ethanol media additives, and incubation temperature. This study is the first to describe simple, reliable protocols for in-vitro germination of *G. philippii* basidiospores. A standardised method for optimum axenic spore germination was described for each species. The germination dynamics of the basidiospores as observed *in vitro* were discussed in relation to their ecology.

The sexuality and mating systems of *G. philippii, G. mastoporum,* and *G. australe* were determined. Observation of somatic interactions between monokaryotic and dikaryotic mycelia using genetically defined material confirmed the incompatibility mechanisms which operate to delimit individuals of these species in a population. Ten monokaryotic siblings; harvested as single spore isolates from each of two *G. philippii*, one *G. mastoporum* and two *G. australe* basidiocarps were paired in every possible combination. The macro- and micro-morphology and nuclear behaviour of interacting individuals were recorded at 7-day intervals. Ratios of ~1:4 compatible to incompatible crosses were observed between monokaryotic *G. philippii, G. mastoporum*, and *G. australe* siblings are consistent with ratios found for other *Ganoderma* species, indicating a tetrapolar mating system with alleles for heterothallism at two loci.

The incidence and severity of red root disease increases with rotation. This may be linked to changes in the mode of dispersal of *G. philippii,* the balance between vegetative and spore dissemination. Preliminary somatic incompatibility tests indicated that genetic diversity increased with successive rotations implying an increasing, not decreasing role of spores. It was decided to further explore this observation with microsatellite analysis.

Primers targeting microsatellite loci were screened against three isolates of *G. philippii* from geographically separate locations. All but two of the primer pairs successfully amplified *G. philippii* DNA, but the majority amplified a single product that did not vary among the test isolates. Eight of the primer sets were polymorphic and heterozygous in at least one of the test isolates.

Populations were genetically distinct with high levels of inbreeding and clonal spread to adjacent trees increased after the first rotation. Despite the high levels of genetic diversity seen at all sampling scales, migration rates appear low. Measures to reduce the under-ground spread of the pathogen as well as methods to prevent the initiation of new infections from basidiospores will be needed to reduce the incidence of root rot in *Acacia mangium* plantations.

The final chapter of the thesis reviews and discusses the significance of the above results in relation the hypothesis tested i.e. red root-rot disease, caused by *G. philippii*, predominantly occurs and spreads in tropical acacia plantations as a function of vegetative (clonal) infection pathways. The contribution of new information generated in this thesis to possible management strategies are discussed and recommendations are given for further research.

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CHAPTER 1. GENERAL INTRODUCTION

1.1. Root-rot disease in plantation forests of SE Asia

In tropical south east (SE) Asia, plantation forests of Australian acacia and eucalypt species now exceed seven million hectares (Harwood and Nambiar 2014). They are managed on short rotations, typically 5–8 years, for wood production (Harwood 2014) and their main purpose is to supply wood for the wood processing industries throughout the region, such as the pulp and paper industry (Harwood and Nambiar 2014). The growing and processing of pulp and paper crops represents a primary source of income for the rural Indonesian communities of Sumatra and Kalimantan especially where alternative income earning opportunities are scarce (ITS Global 2011). Productivity of non-native hardwood plantations is therefore of critical importance to the region, but all indications are that this will be challenged by increasing numbers of disease and pest problems, this being a worldwide trend. These fungi include accidental introductions, originating from the same areas of origin as the trees, as well as 'new encounter' pathogens that are undergoing host shifts as they infect non-native trees (Wingfield *et al.* 2011).

In Indonesia in 2014, three species, *Acacia mangium* Willd. (~0.6M ha), *Acacia crassicarpa* A. Cunn. ex Benth. (~0.3M ha) and *Eucalyptus pellita* F. Muell. and hybrids (>0.3 Mha) accounted for most of the hardwood planted for pulp and paper production because of their good performance (Harwood and Nambiar 2014; Harwood 2014). According to Nambiar and Harwood (2014) growth rates of *A. mangium* plantations in Sumatra ranged between 22 and 35 m3/ha/yr but when impacted by fungal diseases (*Ganoderma* and *Ceratocystis*), this has reduced growth on infected sites to 15 m^3/ha/yr or lower.

Ganoderma is a fungal basidiomycete genus which contains pathogenic species. These species originate in native forest stands which, when cleared for plantation, persist as

saprophytes colonising woody debris such as remnant stumps and roots (Mohammed *et al.* 2014). This debris provides the inoculum for the infection of new plantings (Mohammed *et al.* 2014). Substantial inoculum loads can build up over several rotations of *A. mangium* as the fungus continues to kill trees and to colonise dead wood (Lee 2004; Mohammed *et al.* 2014).

Mortality rates reported appeared relatively low in the first rotation (around 5%), however build-up of the inoculum load in woody debris meant that up to 28% of trees were killed at some sites in Indonesian second-rotation *A. mangium* (Irianto *et al.* 2006). Surveys of mortalities across rotations of different ages have given rise to estate wide values that probably grossly underestimate the impact of root rot on productivity at harvest (Francis *et al.* 2014). In Central Sumatra, by the third rotation, several sites were found to be no longer capable of providing a commercial yield at harvest (Francis *et al.* 2014).

These levels of tree death have driven a shift to growing eucalypts, especially *E. pellita* and its related interspecific hybrids. This change to a different species has been further accelerated by high levels of mortality in *A. mangium* being caused by another disease, *Ceratocystis* wilt (Nambiar and Harwood 2014; Nasution *et al.* 2019; Tarigan *et al.* 2011). In total by 2016–2017 in Sumatra, between 95-98% of *A. mangium* had been replaced by eucalypts (Nambiar *et al.* 2018). The rate and scale of this change are unprecedented and associated with unknown consequences for the economy and ecosystems (Nambiar *et al.* 2018). Switching from acacia to eucalypt is not a universal solution. Eucalypts as exotics are proving equally susceptible to pests and pathogens (Pham *et al.* 2019; Wingfield *et al.* 2015), are limited by type of site and require higher levels of management inputs (vegetation management and fertilizer application) to achieve satisfactory growth rates than do acacias (Harwood and Nambiar 2014).

1.2. Causal agents of root rot disease in SE Asian hardwood plantations

There are several genera of candidate fungi within the Basidiomycota in SE Asia that may act as primary or secondary root-rotting pathogens (Glen 2006a; Glen *et al.* 2014; Mohd Farid *et al.* 2009). The most widely reported fungi associated with red root rot disease of tropical pulpwood are species of *Ganoderma*. Many *Ganoderma* spp. have been described from Indonesia and Malaysia (Smith and Sivasithamparam 2003; Steyaert 1972), and several have come to attention as pathogens of perennial crops established after the clearing of native forests (Glen *et al.* 2009; Hidayati *et al.* 2014). *Ganoderma philippii* (Bres. & Henn. ex Sacc.) Bres. [= *G. pseudoferreum* (Wakef.) Overeem & B.A.Steinm.], *G. mastoporum* (Lév.) Pat., *G. australe* (Fr.) Pat., and *G.* aff. *steyaertanum* (B. J. Sm. & Sivasith), have all been implicated with red root-rot in *A. mangium* (Glen *et al.* 2009; Lee 2004).

Ganoderma philippii has now been recognized as the dominant pathogen causing root-rot disease (as opposed to a suite of *Ganoderma* spp.) in both *A. mangium* and *E. pellita* plantations in Sumatra (Coetzee *et al.* 2011; Glen *et al.* 2009). A fourth species, *G. steyaertanum*, has been reported as similarly pathogenic to *A. mangium*, but to date only in Central Java (Hidayati *et al.* 2014).

1.3. Life cycle of *Ganoderma philippii*

Relatively little of the basic biology of *G. philippii* has been substantiated, although much can be inferred by reference to better-known and understood basidiomycete root rot pathogens. Indeed trials for the management of root rot basidiomycete pathogens in tropical pulpwood crops have drawn heavily from the accumulated knowledge of the biology and management of similar temperate pathogens including species of *Heterobasidion* (Garbelotto and Gonthier 2013; Stenlid and Redfern 1998) and *Armillaria* (Baumgartner *et al.* 2011). Understanding a pathogen however by unsubstantiated inference poses real challenges to developing effective

disease management strategies. For example, the poor performance of the biological and chemical treatments applied by Indonesian acacia growers in attempts to reduce inoculum levels was almost certainly due to their non-specificity resulting, in part, from a poor understanding of the disease organisms and their behaviour in plantation monocultures (Mohammed *et al.* 2012).

Tropical tree crops such as oil palm and rubber were planted decades before acacia and eucalypts and have longer histories of disease management. Growers traditionally considered basidiomycete pathogens of these tropical tree crops to disseminate vegetatively in plantations. The fungus persists saprotrophically in the form of a mycelium within stumps and roots after the host tree dies or is cut down and colonized roots serve as an inoculum source when the roots of replanted trees grow into direct contact and are colonized by this resident mycelium (Chee 1990; Flood *et al.* 2000b; Nandris *et al.* 1987b; Turner 1965). Vegetative or clonal primary and secondary infection pathways of oil palm by *Ganoderma boninense* Pat. were described by Thompson (1933) and remained unchallenged as a principle underpinning disease management practice for over sixty years (Sanderson and Pilotti 1997).

More recently the role of root to root infection has been questioned, based on the high genetic diversity of *G. boninense* isolates (Miller *et al.* 1999; Pilotti *et al.* 2018; Pilotti *et al.* 2003). Recent studies in this oil palm pathosystem have revealed that spore dispersal plays a far more important role (relative to vegetative dissemination) than previously considered especially in the colonization of new habitats (Pilotti *et al.* 2018; Rees *et al.* 2011). Genetic assays, from the determination of mating alleles, somatic incompatibility tests complemented by screening with mitochondrial DNA markers have clearly demonstrated infections from separate genotypes arising through sexual recombination (Miller *et al.* 1999; Pilotti *et al.* 2003). There is now a strong body of evidence to support a much greater influence of basidiospore dispersal (relative to mycelial growth) than previously considered in the

colonization of new habitats (see *G. boninense* and *Armillaria mellea* (Vahl ex Fr.) Quel Rees, 2011; Travadon, 2012).

Extensive surveys conducted in Indonesian and Malaysia have recorded root rot infection in *A. mangium* six months after establishment (Irianto *et al.* 2006; Lee 2000). Infected trees (or disease foci) are randomly distributed but tend to aggregate; tree mortality rates can double in 12 months. New disease centres can arise at any stage of a rotation and become centric to the progressive infection and death of neighbouring trees (Francis *et al.* 2014; Irianto *et al.* 2006). Circular patches increase in diameter leading to large areas of stand mortality (Hidayati *et al.* 2014). These epidemiological characteristics agree with traditional views that vegetative disease spread predominates; also suggesting that the rate of disease spread is dependent on initial disease incidence levels. Taking into account that mature acacia trees can have roots extending over several planting rows, root contact is potentially frequent and that the presence of fruiting bodies does not appear to correlate with the level of disease incidence, most authors concluded that initial root rot disease occurred from contact with residual inoculum in the debris left from the harvest of native forest (Mehrotra *et al.* 1996). They also inferred from the more rapid and higher levels of tree mortality with each consecutive rotation that the inoculum load builds up in stumps and other debris with successive rotations (Mohammed *et al.* 2014). However, new disease centres can arise in-between disease gaps and in stands with no previous disease history, at any stage of the rotation cycle (Francis *et al.* 2014). New infections of mature trees ostensibly spatially isolated from secondary infection courts also imply that although the spread and infection of *G. philippii* probably occurs clonally, there may be significant input into these processes from basidiospores (Mohammed *et al.* 2012).

1.4. Elucidating pathogen mode(s) of spread

The relative importance of new spore infections versus mycelial spread from pre-existing infections is said to be reflected by the number and size of genetic individuals in a stand. High numbers of individual mycelia per unit area implies frequent spore (sexual) infection and dispersal events, while the relative size of each (clonal) genet per unit area reflects the extent of vegetative transmission (Stenlid and Redfern 1998).

Mechanisms of vegetative self-nonself recognition (termed vegetative incompatibility, mycelial incompatibility, or somatic incompatibility, particularly in basidiomycetes) were first suggested from reactions between isolates of the same species from different origins, when paired in axenic culture (Esser 1962; Ratkowsky 1986). In basidiomycetes, recognition has a genetic basis and is regulated by one to three or, possibly more genes with multiallelic loci (termed vegetative compatibility (VC), vic or het loci). With basidiomycetes, most mycelia (usually dikaryons) isolated from the natural environment tend to be incompatible when paired, implying that vegetative incompatibility groups usually correspond to genetic individuals, though this is not true for all species. Pairing tests of intraspecific somatic incompatibility (SI) have been used as a guide to identifying genetically distinct isolates within populations of several economically important root and wood rotting basidiomycete species (Guler 2008; Latiffah and Ho 2005; Marcais *et al.* 2000; Pilotti *et al.* 2003; Prospero *et al.* 2008; Prospero *et al.* 2003; Suwandi *et al.* 2004). Discrete mycelia, identified on this basis as being members of one group, are referred to as constituting clones of the same compatibility genotype (Hougaard 1985).

These vegetative somatic incompatibility studies do not always give reliable information. For example, early such studies with *G. boninense* isolates attributed a significant role to vegetative dispersal (Miller 1995; Miller *et al.* 1999). It is now known that basidiospores are

the primary means of spread with direct basidiospore infection via cut stem surfaces made by the pruning of fronds and harvesting of bunches (Rees *et al.* 2009). Much of this new knowledge arose through the application of molecular tools e.g. Pilotti et al 2018.

While molecular markers have been used to detect, identify, and measure detailed differences among subpopulations of temperate forest pathogens such as *Armillaria* (Abomo-Ndongo *et al.* 1997; Prospero *et al.* 2003; Wargo and Shaw III 1985) and *Heterobasidion* (Chase and Ullrich 1983; Garbelotto and Gonthier 2013; Pukkala *et al.* 2005), and tropical root rots in rubber (Liyanage *et al.* 1980; Nandris *et al.* 1988; Suwandi *et al.* 2004) and oil palm (Cooper *et al.* 2011; Pilotti *et al.* 2002; Rees *et al.* 2011), this information is unknown for *G. philippii.*

1.5. Sexuality: breeding systems and mating types genes

The sexual behaviours of *G. philippii*, *G. mastoporum* and *G. australe* were unknown at the start of this doctoral study. Fungi have mating systems that regulate the amount of heterogeneity that will be present in the next generation. Some fungi are self-fertile but many fungi have genetic systems that prevent mating between very genetically similar individuals. Compatible matings are determined by mating-type factors (MAT) factors. Although MAT loci are complex they are usually treated as if they are simple alleles at two loci with multiple alleles (Burnett 2003; Stenlid 2008a; Worrall 1999).

Some of the species within Basidiomycota have the most complex systems of sexual reproduction known among fungi and have been described by many authors (Adaskaveg and Gilbertson 1987; Angwin 1989; De 1980; Hseu and Wang 1996; Kües *et al.* 2011; Pilotti 2001; Tomsovsky and Homolka 2004; Triratana and Chaiprasert 1991).

Almost 60% of heterothallic basidiomycetes are characterised by a complex bifactorial incompatibility system, involving two mating-types with many alleles. In this system two

factors, A and B, together determine mating competence and two individuals will only be compatible if they have different alleles at both A and B loci. These factors are generally on separate linkage groups and their independent segregation at meiosis leads to the production of four main types of progeny. Basidiospores can possess nuclei that have the same mating factors as either of the parents (i.e. A_1B_1 or A_2B_2), and A_1B_2 and A_2B_1). Systems with four possible combinations of mating factors are termed tetrapolar.

The mating systems of *Ganoderma resinaceum* Boud, *Ganoderma colossus* (Fr.) Torrend (De 1980), *Ganoderma lucidum* (Curtis) P. Karst. (Triratana and Chaiprasert 1991), *Ganoderma tsugae* Murr., *Ganoderma microsporum* Hseu (Hseu and Wang 1996), and *G. boninense* (Pilotti 2005; Pilotti *et al.* 2002) have all been determined as heterothallic and tetrapolar with multiple alleles at both mating-type loci.

1.6. Silviculture, spore dispersal and colonisation

Although basidiomycete root rot pathogens are present in unmanaged forest, serious disease is often confined to managed forests in which regular thinning takes place (Eyles *et al.* 2008). Newly created stumps provide the optimum avenue for infection by spores, but the period of susceptibility is relatively short (Lim 1977; Miller *et al.* 1999). Infection may also be limited by the availability of moisture, temperature and by competition from other organisms (Page *et al.* 2017).

The principal source of inoculum for infection by the northern hemisphere root rot pathogen *H. annosum* is provided by colonised stumps and infected tree roots; direct infection of living trees by spores is rare (Stenlid and Redfern 1998). The relative epidemiological importance of spores and infected debris varies during the lifetime of a crop and with intensity of management techniques, especially thinning which creates stumps during the rotation and avenues for spores (Stenlid and Redfern 1998). The colonisation of stumps by spores of *H. annosum* clearly has

great epidemiological significance therefore in plantations newly established on land where woody inoculum is lacking and all inoculum originates from stump colonisations (Stenlid 2008a). In successive rotations of plantations established on land without a previous forest history an increasing proportion of stumps from felled trees will be occupied by the pathogen (Stenlid and Redfern 1998). Information from modelling infection and disease spread (Thor *et al.* 2005) and the analysis of stump sampling (Piri 1996) suggest that the balance changes and below ground spread assumes a greater importance although some stumps will remain available for spore infection. Although the importance of spore-infected stumps as a direct cause of damage can be expected to decline with time, it will continue to have significance for genetic diversity (Woodward *et al.* 1998).

1.7. Management strategies and research direction

There are no published guidelines for the management of root-rot disease in *A. mangium* plantations. However, the approaches currently under investigation are like those that have successfully controlled root-rot disease in other plantation systems (Eyles *et al.* 2008; Mohammed *et al.* 2014).

Biological control of root-rot fungi may be achieved using non-pathogenic lignolytic basidiomycetes, fungi that can break down woody debris, occupy the same resource as the pathogen, compete for nutrients, produce inhibitory compounds, or are mycoparasites (Hill *et al.* 2010). Their potential for use as a natural means of reducing inoculum while outcompeting pathogens represents an attractive alternative to less environmentally friendly, chemical-based control (Hushiarian *et al.* 2013). However, the only biological control agent (BCA) currently commercially available for basidiomycete root-rot disease is *Phlebiopsis gigantea* (Fr.) Jülich (Rönnberg *et al.* 2006). Used throughout Europe and North America for the control of *H. annosum* in both temperate pine and spruce, the commercial formulation was developed

over many years (Stenlid and Redfern 1998). Biological control of *H. annosum* by *P. gigantea* is achieved by preventing basidiospore colonisation through host stumps with an application of BCA oidia (asexual spores) that outcompete the pathogen (Pratt *et al.* 1999). Three distinct biological control products based on *P. gigantea* have been developed: PG Suspension in the UK, PG IBL in Poland and Rotstop in Finland (Pratt *et al.* 2000). Application by a mechanical harvester is now the predominant application system (Pratt *et al.* 2000).

Indonesian and Malaysian pulpwood growers are currently focussing their research and development efforts on selecting for genetic resistance and biological-control using nursery inoculated endophytes, although with limited success (Mohammed pers. comm.). These strategies are more likely to be deployed with success over the many thousands of hectares involved in tropical plantations, especially in SE Asia where topography and climate often prohibit other management approaches such as stump removal and chemical control.

1.8. Objectives and methodology of this research

Ganoderma philippii root-rot poses substantial and intractable management challenges for the SE Asian pulpwood plantation industry (Mohammed *et al.* 2014). The challenge for forest managers is to better understand the dynamics of the disease, as this information underpins risk, impact assessment, and the development and deployment of management strategies (Laflamme 2010; Redfern *et al.* 2010).

The main hypothesis tested for *G. philippii* was;

Red root-rot disease, caused by *G. philippii*, predominantly occurs and spreads in tropical *A. mangium* and *E. pellita* plantations as a function of vegetative (clonal) infection pathways.

To answer this hypothesis my study comprised three experimental investigations to help provide information to support or reject the hypothesis;

1. *Ganoderma* basidiospore germination

Very little was known about the germination ability of *G. philippii* basidiospores but the axenic germination of *G. philippii* basidiospores has been problematic (Lim 1977). The only previously published study on the germination of *G. philippii* basidiospores (as *G. pseudoferreum*) reported as necessary to germination the ingestion and passage through the gut of larvae of two species of the Tipulid fly *Limonia*, a normal constituent of the micro-fauna of its basidiocarps (Lim 1977). We collected sporocarps of different *Ganoderma* species from across Indonesia and Malaysia, and also developed a technique to harvest the spores from sporocarps in situ (it is difficult to avoid contamination). We compared environmental factors influencing germination between the pathogenic species *G. philippii* and two saprophytic species *G. australe* and *G. mastoporum* that are frequently found fruiting in tropical acacia and eucalypt plantations. Protocols for the germination and storage of single spores and their isolates was also developed.

The ability to collect and germinate *G. philippii* basidiospores will also serve to facilitate later *in vitro* experiments in wood colonisation by *G. philippii* basidiospores and stump or simulated stump experiments to determine the effectiveness of BCAs in preventing colonisation by basidiospores.

2. *Ganoderma* mating systems

A knowledge of the breeding system of *G. philippii* will support the study of population genetics, indicating the epidemiological role of spores. To assess the breeding system of *G. philippii*, an analysis of the pairing behaviour between sibling monokaryons and dikaryon - monokaryotic pairings was carried out. The breeding systems of the two saprotrophic *Ganoderma* species *G. australe* and *G. mastoporum* were also examined.

3. The population structure of *G. philippii* in *Acacia* and *Eucalyptus* plantations.

The incidence and severity of red root disease increases with rotation. This may be linked to changes in the mode of dispersal of *G. philippii* and shifts in the balance between vegetative and spore dissemination. Preliminary SI tests indicated that genetic diversity increased with successive rotations (Mohammed *et al.* 2012) implying an increasing, not decreasing role of spores. An incompatible SI reaction only indicates that two isolates are not genetically identical. Analysis of genetic diversity based on microsatellites allowed a more accurate determination of relationships among isolates within disease centres and among neighbouring centres.

At the start of the doctoral study an Indonesian pulp and paper company offered two of their ex-experimental sites (at Baserah and Logas in Sumatra) for a microsatellite study. The company had used these sites to investigate the susceptibility of 4 hardwood species (2 acacia and 2 eucalypts) and the influence of destumping and stump retention. As both sites were to be harvested at the start of the current project and replanted, the plan was to sample before harvest and in the replanted plantations of *E. pellita* at Baserah and *A. mangium* at Logas. Also available were many isolates (~500) obtained from 7 other sites in Sumatra. Access to this diverse set of isolates mean that the genetic structures of different populations of *G. philippii* could be analysed and both the role of spore dispersal and the influence of certain silvicultural management regimes on genetic diversity explored. It was not however possible to compare different rotations at the same site but only the level of variation at different sites with different rotation numbers.

1.9. Thesis Structure

The University of Tasmania encourages doctoral candidates to present doctoral research in the style of ‘thesis by publication’. Hence, subsequent chapters in this thesis are presented in the form of stand-alone scientific journal papers.

Chapter 2. Basidiomycete root-rot diseases in South East Asian forest plantation crops: a review

The introduction and literature review of this thesis contains excerpts from Mohammed, C.L., Rimbawanto, A. & Page, D.E. 2014. Management of basidiomycete root- and stem-rot diseases in oil palm, rubber and tropical hardwood plantation crops. *Forest Pathology*, 44, 428-446. (Appendix 1).

Caroline Mohammed (75%), Anto Rimbawanto (5%), David Page (20%). Caroline Mohammed structured and wrote the review, David Page searched for information especially grey material which he collated and drafted into different sections. Anto Rimbawanto was the Indonesian partner and leader of the research and capability building project under for which this review was written and approved the final draft on behalf of Indonesian collaborators.

Other literature not pertaining to the management of basidiomycete root-and stem-rot diseases in tropical plantation crops has also been included to support the approach taken in the thesis in respect to the main hypothesis. This includes information about spore germination and dispersal, the vegetative and sexual behaviour in basidiomycetes and the possible application of this knowledge, the application of cultural and molecular techniques to the investigation of fungal populations, case studies of what is known for saprophytic and pathogenic fungi in natural forests and tree plantations, the relevance of the knowledge in the literature examined to the management of basidiomycete root-rot pathogens.

Chapter 3. *Ganoderma* basidiospore germination responses as affected by spore density, temperature and nutrient media.

This study is the first to describe simple, reliable protocols for *in-vitro* germination of *G. philippii* basidiospores. The germination dynamics of *G. australe*, *G. mastoporum* and

G. philippii basidiospores under different nutritional and physical conditions are empirically modelled in order to facilitate the development of single spore isolation methods for use in subsequent sexual studies. Concurrently, key physiological and ecological factors are identified from a putatively representative set of germination conditions thought likely to be encountered in Indonesian pulpwood stands, that either alone or in combination, are predictive of *Ganoderma* germination.

David Page (77.5%) designed and conducted the study, data analyses and wrote the manuscript. David Ratkowsky (10%), Morag Glen (7.5%), Chris Beadle (2.5%), Caroline Mohammed (2.5%) contributed to design, data collection and analyses, and edited the manuscript. Anto Rimbawanto (2.5%) facilitated the industry permissions for, and logistics of, data collection and access to laboratory facilities. This chapter was published as is presented in the thesis.

Page DE, Glen M, Ratkowsky DA, Beadle CL, Rimbawanto A, Mohammed CL, 2017. *Ganoderma* basidiospore germination responses as affected by spore density, temperature and nutrient media. *Tropical Plant Pathology* 42, 328-38.

Chapter 4. Sexuality and mating types of *Ganoderma philippii, Ganoderma mastoporum* and *Ganoderma australe,* three basidiomycete fungi with contrasting ecological roles in south-east Asian pulpwood plantations.

Basidiospores from two sporocarps of *G. philippii*, two sporocarps of *G. australe* and one sporocarp of *G. mastoporum* were germinated and monokaryon isolates obtained. Ten monokaryons from each sporocarp were paired in all sibling combinations and scored for formation of clamp connections and nuclear migration by fluorescent microscopy.

David Page (75%) designed and conducted the study, data analyses and wrote the manuscript, Morag Glen (10%), Anto Rimbawanto (2%), Caroline Mohammed (2%), David Ratkowsky (2.5%) contributed to design, data collection and analyses, and edited the manuscript. Desy Puspitasari (1%) supported the laboratory work and maintenance of cultures. This chapter was published as is presented in the thesis.

Page DE, Glen M, Puspitasari D, Rimbawanto A, Ratkowsky D, Mohammed C, 2018. Sexuality and mating types of *Ganoderma philippii*, *Ganoderma mastoporum* and *Ganoderma australe*, three basidiomycete fungi with contrasting ecological roles in south-east Asian pulpwood plantations. *Australasian Plant Pathology* 47, 83-94

Chapter 5. *Acacia* plantations in Indonesia facilitate clonal spread of the root rot pathogen, *Ganoderma philippi*.

The availability of next-generation sequencing technologies has expedited the discovery of microsatellite loci for population genetic studies without prior DNA sequence knowledge. In this study, DNA was extracted from *G. philippii* mycelium and sequenced on a Roche GS FLX. Shotgun sequences were screened for simple sequence repeat motifs of 2-6bp, with at least four repeats, producing a file of 856 potential microsatellite sequences. A subset of primers were screened against three isolates of *G. philippii* from geographically separate locations to select primers for the study. The population genetics of *G. philippii* was then studied to evaluate the role of sexual and asexual reproduction in its mode of spread.

David Page (75%) designed and conducted the study, data analyses and wrote the manuscript, Morag Glen (10%), Caroline Mohammed (5%) contributed to design, data collection and analyses, and edited the manuscript. Abdul Gafur, Anto Rimbawanto, Istiana Prihatini, Desy Puspitasari provided a total contribution of 10% in industry support and/or contributed to the collection, isolation and maintenance of isolates and/or isolate identification

and DNA extraction. The chapter is a slightly expanded version of the paper recently accepted by Plant Pathology as this journal imposes a strict word limit.

Page DE, Glen M, Puspitasari D, Prihatini I, Gafur A, Mohammed CL. 2019. *Acacia* plantations in Indonesia facilitate clonal spread of the root pathogen, *Ganoderma philippii*. *Plant Pathology* 69, 685-697.

Chapter 6. Synthesis of results and implications for management

The results of the study are discussed in relation to possible management strategies, such as tolerance and biocontrol, as these are the most likely to be deployed with success over the many thousands of hectares involved in tropical plantations.

CHAPTER 2. BASIDIOMYCETE ROOT-ROT DISEASES IN SOUTH EAST ASIAN FOREST PLANTATION CROPS: A REVIEW

2.1. General introduction

2.1.1. Tropical hardwood industry in SE Asia and economic importance

A range of Australian acacia and eucalypt species have been widely planted in plantations and smallholder woodlots throughout SE Asia (Harwood 2014). Areas of acacia and eucalypt plantations have expanded in SE Asia during the last two decades and there are now at least 2.6 million ha of acacias and 4.3 million ha of eucalypts (Harwood 2014). These plantations are managed in short rotation cycles typically of 5–8 years, with large areas moving into successive rotations (Nambiar *et al.* 2015). They are mainly managed to supply wood for the processing industries, mostly pulp and paper production. However, use for sawn timber, veneer and composite products (such as medium-density fibreboard) is increasing, for example large volumes of acacia wood are sawn for furniture making in Vietnam (Nambiar *et al.* 2015). Poles for construction work and firewood are other important uses (Nambiar and Harwood 2014). With over 7 M ha now planted, these non-native trees are an important part of the wood supply chain for Asian forest industries and generate very substantial livelihoods for smallholders (Nambiar and Harwood 2014).

Indonesia's forest industries are dominated by the pulp and paper sectors. The pulp and paper industry accounts for 6.7% of Indonesia’s gross domestic product generated by components of processing industries, employs 260 000 workers directly and 1.1 million workers indirectly, and in 2016, it ranked as the country’s seventh largest foreign exchange earner contributing US$3.8 billion (Nambiar *et al.* 2018) and contributes around 1.8% of GDP (FAO 2016). Two species, *Acacia mangium* and *Eucalyptus pellita* and hybrids account for the majority of the plantings for pulp and paper on mineral soils because of their rapid growth, although there is

significant smallholder interest in higher value species and products, especially in Java (Nambiar and Harwood 2014). As part of the need to place the pulp and paper industries onto a more sustainable footing and to safeguard native forest from illegal logging, Indonesia's Ministry of Forestry promotes policies that encourage the development of a plantation-based wood supply (Ministry of Forestry 2007). For example, a provisional moratorium on issuing forest-clearing permits for plantations and logging (Gilbert 2012) has been made permanent (Ahluwalia 2019). Establishment and maintenance of large areas of sustainably productive plantations is recognised as the only way to minimise clearing of the remaining natural forest, as the huge pulp capacity and planned expansion will draw its feedstock either from plantations or from the natural forest (Nambiar *et al.* 2018). Community and market concerns about widespread deforestation (Stibig *et al.* 2014) have had significant impact.

In addition to the pulp and paper companies, plantations are also owned and managed by many small to medium wood growing enterprises. The economic and social impacts of this sector in Indonesia are important. In some regions such as Riau in Sumatra the net impacts of the sector on economy, tax revenue, employment (with high job multiplier effects) and household income are substantial and critical ITTO (2017).

2.1.2. Australian acacias and eucalypts in SE Asia

Acacia mangium is native to northern Queensland, Australia, mostly in the coastal tropical lowlands. The species also occurs naturally in the Western Province of Papua New Guinea and the Indonesian provinces of Papua (Manokwari, Merauke) and Maluku (Seram, Aru) (Butcher *et al.* 1998). It occurs naturally along tropical warm and hot climatic boundary zones, along the fringes of rain forest, open forest and woodland and will occur on a wide range of soil types derived from acidic parent material, even those with poor drainage and low fertility (Pedley 1986).

The first introduction of *A. mangium* for use as a plantation species in south east Asia was into Sabah, Malaysia in 1966 by the State Forest Department (Harwood 2014). The seed was originally collected from a single tree at Mission Beach (Queensland). Its performance in these first plantings and subsequent trials was promising (Liang 1986). Of note was its ability to compete successfully with the shade intolerant grass *Imperata cylindrica* (lalang grass) on poor soils which were often degraded abandoned farmland. Therefore, the species was subsequently used for the afforestation of grasslands and abandoned farmland areas. Planting for commercial plantations on a large scale started in Malaysia in 1976 (Udarbe and Hepburn 1986) and it quickly became a popular species in many countries, including Indonesia, Thailand, Vietnam and the Philippines (Pinyopusarerk *et al.* 1993).

Indonesia introduced *A. mangium* as a potential plantation species in 1979, first in Sumatra and later in Kalimantan (Old *et al.* 2000). Seeds consisting of three seedlots were brought from Sabah and used in a field trial in Subanjeriji, South Sumatra (Old *et al.* 2000). The species trial confirmed the superiority of *A. mangium* over other acacia species, due to its good characteristics as a legume, being adapted to, and growing well on, the inherently acidic and poor red-yellow podsolic soils dominating the company plantation sites and in a lowland humid environment (Otsamo 1996). Arisman (2006) reports that following the successful species trial in Subanjeriji, seed from four provenances of Queensland's Cairns region, namely Cassowary, Jullaten, Mossman and Daintree, were imported in 1980. Provenance resource stands, based on offspring of 10 individual parent trees from each location, were established in Subanjeriji (300 ha) and Benakat (100 ha) using these new Australian provenances and are referred to as the Subanjeriji local landrace. In 1982, seeds collected from Sidei (Manokwari), Seram (Maluka), Sanga-Sanga (East Kalimantan) were also introduced into Subanjeriji, but the growth of these provenances was poor. As such the Subanjeriji provenance stands were used as seed production areas. First-rotation *A. mangium* plantations yielded 20–35 m^3 ha^{-1} y^{-1} in 8–10-year rotations

(Harwood 2014). The species did not require much tending and at this stage was relatively free of pests and diseases. Encouraged by this, companies expanded *A. mangium* plantations with the expectation that they would be the primary source of pulp wood for several large pulp mills (Harwood 2014). Large-scale planting of *A. mangium* has been in progress in Sumatra since 1990, and the area increased from 600,000 hectares in 1988–89 to 800,000 hectares in 2011 (Harwood 2014). Two fungal diseases (*G. philippii* and *Ceratocystis manginecans*) have since become epidemic and rendered *A. mangium* plantations unviable in Sumatra (Harwood 2014) and increasingly so in Kalimantan. Industry, faced with this serious and largely unanticipated threat to wood production on mineral soils and without effective control measures, rapidly changed species from *A. mangium* to *E. pellita* and related interspecific hybrids. The rate of replacement in one company reached more than 50 000 ha y−1. In total, more than 600 000 ha had been replaced by 2016–2017 (Nambiar *et al.* 2018).

Eucalyptus spp. (Myrtaceae) have also been planted over large areas of SE Asia for pulpwood production. It is estimated that eucalypt plantations in the south east Asian region now exceed 2.0 million ha, 120,000 ha of which are in Indonesia (Nambiar and Harwood 2014). Most plantations are in Sumatra (Aceh, North Sumatra, Jambi) and Kalimantan (West, East and South Kalimantan). The main species planted are *Eucalyptus deglupta* and *Eucalyptus urophylla*, which are native to Indonesia and Malaysia, although their natural distribution is in the eastern islands. Many other species and hybrids have been tried in small-scale experimental plantations, notably, *Eucalyptus camaldulensis*, *Eucalyptus grandis*, *E. pellita*, *Eucalyptus tereticornis* and *Eucalyptus torelliana* (Nair 2000). Experimental plantings of *E. pellita* were undertaken in Indonesia in the mid-1990s (Nair 2000). The species is endemic to north-eastern Queensland. Genetic trials of *E. pellita* established that in the low-elevation environments of Sumatra, provenances from Papua New Guinea and West Papua grew better than Queensland provenances. Some growers now produce *E. pellita* seed from

seed orchards based on these selected provenances, and individual clones of *E. pellita* have been developed. Today, roughly 80% of *E. pellita* plantations established in Indonesia are clonal (Hardiyanto and Wicaksono 2008). The growth potential of *E. pellita* was considered lower than that of *A. mangium* grown at the same site (Hardiyanto and Wicaksono 2008). However, the consideration of growth potential did not consider the loss of productivity due to pests and diseases with *A. mangium*.

There are many possible causes of damage and mortality in any tree plantations such as damage from termites, elephants, squirrels and monkeys, insect pests, fungal diseases, wind-throw or flooding. Forest trees planted as non-natives in various parts of the world are increasingly threatened by insect pests and pathogens as plantation estates age, especially in south east Asia (Nambiar *et al.* 2015; Nambiar *et al.* 2018; Tarigan *et al.* 2011; Wingfield *et al.* 2001; Wingfield *et al.* 2011; Wingfield *et al.* 2008). These include those that are introduced accidentally, as well as 'new encounter' insect pests and pathogens that are undergoing host shifts (Eyles *et al.* 2008). In SE Asia one of the most important groups of fungal pathogens that have shifted to exotic plantation hosts are basidiomycetes (*Ganoderma*, *Rigidoporus* and *Phellinus* spp.), which cause root-rot disease (Ariffin *et al.* 2000; Chee 1990; Eyles *et al.* 2008; Lee 2000).

2.1.3. Impact of basidiomycete root rot pathogens to tree crops in SE Asia

Basal stem rot and root rot in trees caused by basidiomycetes are naturally and widely occurring diseases on a wide range of hosts, and have historically been one of the most intractable problems confronted in commercial forestry and tree plantation industries worldwide (Ariffin *et al.* 2000; Chung 2011; Durand-Gasselin *et al.* 2005; Holdenrieder *et al.* 2004; Hood 2006; Hood and Kimberley 2009; Korhonen and Stenlid 1998; Mohammed *et al.* 2014; Pratt 1998; Shaw *et al.* 1989; Stenlid and Redfern 1998). They can cause significant

mortality, and are a common explanation for failure in the early phase of plantation development (Wingfield *et al.* 2001). Trees in tropical rainforests are hosts to a range of stem- and root-rot pathogens, typically of the basidiomycete genera *Phellinus*, *Rigidoporus* and *Ganoderma* (Lee 1997). In particular, basidiomycete root rot is a major threat to plantation monocultures that have been established on land converted from natural forest with poor land-clearing techniques (Mohd Farid and Lee 2006). Furthermore, low species and genetic diversity, and the uniform age typical to plantation stands create conditions favourable for the development and spread of root rot pathogens (Mohd Farid and Lee 2006).

Root rot caused by *Rigidoporus microporus* (Sw.) Overeem is the most destructive disease of rubber and can kill the tree irrespective of age or health status, causing economic losses to the latex industry. In young rubber plantations in Malaysia, it is responsible for more losses than those caused by all other diseases and pests combined (Johnston 1989). In Sri Lanka, the average loss of rubber stands to root rot is approximately 10% (Liyanage 1997). Losses have reached upwards of 50% in some plantations (Guyot and Flori 2002; Nandris *et al.* 1988). In Indonesia, mean annual incidence has been reported in rubber as 5–15% across all age classes surveyed; the worst affected blocks of 3-year-old plantings had 30% incidence. Levels of incidence in smallholdings were the highest, up to 40–60% (Soepena *et al.* 2000). Such areas would require replanting as early as 20 years after establishment. Root rot disease has also been reported to aggressively kill teak in PNG, Java and Tanganyika, and many species of fruit tree in Taiwan (Ann *et al.* 2002; Ann *et al.* 1999; Mohd Farid *et al.* 2005; Mohd Farid and Lee 2006).

In oil palm, the most damaging basidiomycete disease is referred to as basal stem rot (BSR) and is caused by *G. boninense* in the roots, basal stem and upper stem (Flood *et al.* 2000b). Basal stem rot constrains sustainable production of oil palm throughout Asia (Ariffin *et al.* 2000; Durand-Gasselin *et al.* 2005; Flood *et al.* 2000b; Singh 1991; Turner 1965). Chung (2011)

states that the death of an oil palm tree at age ten years signifies a 15-year loss of production equivalent to 600 kg of crude palm oil and loss of value of approximately $US 675 or $45 per annum. A survey was carried out in Malaysia in 2009–2010 of 1.6 Mha of oil-palm plantation representing approximately 1000 estates (Idris 2012). Nearly 60% of the estates reported the presence of BSR; the average mortality rate was 3.7% which was equivalent to a loss in value of $US 570M per annum. Certain regions experienced much higher than average mortality rates of up to 10%. The following is a general observation about increasing disease incidence across oil palm rotations in Indonesia (Susanto *et al.* 2009):

- 1st rotation: Disease surfaces at about age 10 years; disease incidence at end of rotation (approximately age 25 years) <2%.
- 2nd rotation: Disease centres develop as early as age 6 months; patches of dead trees will develop; disease incidence at end of rotation normally <10%.
- 3rd rotation: Disease centres develop as early as age 6 months; up to 70% mortality at end of rotation in peat soils but as low as 20% in mineral soils.

In India, Indonesia, Malaysia, Sri Lanka, Thailand and the Philippines; basidiomycete root rots have been identified as the most serious disease in plantations of tropical acacias (Old *et al.* 2000). Although individual acacia trees in the host crop suffering from stress are considered more susceptible to attack than vigorous trees (Sariah 2003), apparently vigorous individuals can also succumb to disease within a short period of being visually healthy as observed by Francis et al. (2014). Root-rot disease build-up in acacia and eucalypt plantations is rather like that observed in oil palm and rubber plantations from one rotation to the next. However, because pulpwood rotations are generally only 5 to 6 years, compared with 25 years for oil palm and rubber, there is accelerated disease development, such that in some areas, tree death can exceed 50% within 20 years of establishing the first pulpwood plantation (Irianto *et al.* 2006). Growth rates of *A. mangium* in Sumatra, where most of the industrial plantations have been established, ranged between 22–35 m^3/ha/years before being impacted by fungal diseases

(*Ganoderma* root rot and *Ceratocystis* canker and wilt) (Harwood and Nambiar 2014; Harwood 2014; Nambiar *et al.* 2015). Studies have shown that smallholders currently achieve yields of only around 50% of potential yield from their first-rotation plantations, due to lack of knowledge and/or application of appropriate management, including disease management (Mohammed *et al.* 2014).

2.2. *Ganoderma* diseases of woody perennial crops

There are several candidate basidiomycete fungi that may act as primary or secondary root-rot pathogens in acacia, but the basidiomycete fungi most frequently reported in root rot affected acacia plantations are *Ganoderma* spp. (Bakshi 1957; Boa and Lenné 1994; Glen *et al.* 2009; Lee 1993; Lee 2000; Mehrotra *et al.* 1996; Old *et al.* 2000).

2.2.1. Taxonomy of *Ganoderma* species

Precise identification to species level can be difficult because of the high degree of morphological plasticity and unresolved taxonomic issues within the Ganodermataceae (Yuskianti *et al.* 2014). The family Ganodermataceae was presented by Donk (1948) with *Ganoderma* (Curtis) P. Karst as type and is currently classified in the order Polyporales or "polypores" i.e., Agaricomycotina that grow saprobically or parasitically on wood of seed plants and can form conspicuous, bracket-like sporocarps. Justo *et al.* (2017) considered this family a synonym of Polyporaceae despite its apparent monophyly, though this has not been widely adopted (e.g. Loyd *et al.* 2019). The family designation of Ganodermataceae would require circumscription of another four families consisting of genera currently within the Polyporales, for which there is phylogenetic support but no supporting morphological characters, *Ganoderma* has for some time been regarded as an important, but also very difficult genus among the "polypores" (Costa-Rezende *et al.* 2017; Justo *et al.* 2017; Moncalvo and Ryvarden 1997; Ryvarden 1993). The difficulties have arisen because of morphological

plasticity and have been exacerbated by collections composed of multiple species (Steyaert 1972).

The Ganodermataceae includes seven currently accepted genera (Costa-Rezende *et al.* 2017), though *Ganoderma* is by far the most diverse. The genus *Ganoderma* was created by Karsten (1881) to accommodate a single species, *Ganoderma lucidum* (Curtis) P. Karst.

Ganoderma has been divided into two subgenera: subgen. *Ganoderma*, comprising laccate species with a palisade of inflated hyphae at the pileus surface that give a shiny appearance, and subgen. *Elfvingia* for non-laccate species where the palisade of club-shaped cells is absent in the surface of the basidiomes (Imazeki 1952). Early phylogenetic analyses based on DNA sequence data appeared to support this division (Moncalvo and Buchanan 2008), however more recent analyses including a broader selection of species and based on multigene phylogenetic analyses do not. The type species of *Ganoderma*, *G. lucidum*, was previously known as *Polyporus lucidus* (Curtis) Fr. and the basionym, *Boletus lucidus* Curtis, dates back to the late 18th century. The British mycologist Curtis originally described material that was collected from hazel (*Corylus avellane* L.) in Peckham Common, an area of south London, but the type specimen is apparently lost. Nevertheless, it is important to note that *G. lucidum* is clearly a taxon of European origin, which has strong implications for the taxonomic validity of the Asian traditional medicinal mushrooms referred to as *G. lucidum* (Richter *et al.* 2015).

Ganoderma applanatum (Pers.) Pat. (synonym *Elfvingia applanata* (Pers.) P. Karst.; basionym *Boletus applanatus* Pers., the type species of *Elfvingia*) is considered to have a worldwide distribution. The type material originates from Europe and is preserved at the Rijksherbarium Leiden, Netherlands. Species of subgenus *Elfvingia* often produce rather large and conspicuous basidiomes and are thus much more frequently encountered in the field than

those of subgenus *Ganoderma*, but much less cultivated for medicinal purposes (Moncalvo and Ryvarden 1997).

Ganoderma australe can be distinguished from *G. applanatum* by having larger basidiospores (Moncalvo and Ryvarden 1997). *Ganoderma australe* is common in the tropics and has never been recorded from Europe. The type specimen of *G. australe* no longer exists and the only material deposited in the Royal Botanic Gardens Kew under that name is unlikely to correspond to the original collection (Ryvarden 1993). *Ganoderma lipsiense* (Batsch) G.F. Atk., also belongs to this complex, and has been treated by some authors as the correct name for *G. applanatum.* Some years ago, a proposal to conserve the basionym of *G. applanatum* (as *Boletus applanatus* Pers.), against the earlier name, *Boletus lipsiensis* Batsch was approved by the Nomenclature Committee for Fungi to settle this problem. Therefore, *G. applanatum* is the correct name for this taxon (Richter *et al.* 2015).

2.2.2. Morphological characteristics of *Ganoderma* species

Ganodermataceae can be distinguished macroscopically from morphologically similar basidiomycete genera with bracket-like basidiomes, such as *Fomes* and *Fomitopsis*, by its brown (vs. white) spore deposit. Within the Ganodermataceae the distinction between the genera is sometimes not simple when comparing macro-morphological characteristics. The shape of the basidiome and substrate attachment are of rather restricted value for taxon delimitation in the Ganodermataceae; however, basidiome consistency, weight, and thickness as well as the presence and attachment of the stipe are in some cases valuable for macro identification. For example, within *Ganoderma*, *Ganoderma atkinsonii* H. Jahn, Kotl. & Pouzar, *Ganoderma colossus* (Fr.) CF Baker, *Ganoderma nevadense* Murr., and *Ganoderma oregonense* Murr. have light, spongy basidiomata, while *Ganoderma zonatum* Murr. and *Ganoderma pulverulentum* Murr. have light, woody-corky basidiomata.

Ganoderma mexicanum Pat., *Ganoderma sessile* Murr., *Ganoderma sessiliforme* Murr., and *Ganoderma subincrustatum* Murr. have relatively light, woody basidiomata, while *Ganoderma oerstedii* (Fr.) Murr., has heavy, woody basidiomata. *Ganoderma colossus, G. oregonense, G. oerstedii, G. sessile,* and *G. zonatum* are generally robust, while *G. mexicanum, G. parvulum, G. sessiliforme,* and *Ganoderma weberianum* (Bres. & Henn. ex Sacc.) have slender basidiomata (Torres-Torres and Guzmán-Dávalos 2012).

In general, the spores appear a reliable microscopic characteristic for separating different genera within Ganodermataceae (Welti and Courtecuisse 2010). For instance, *Humphreya* has peculiar spores with a distinct reticulate alveloid or honeycomb pattern (Ryvarden 1980). *Amauroderma* can also be distinguished from *Ganoderma* by its non-truncate spores as well as the habit of growing on the ground out of buried roots (Ryvarden 2004).

According to Ryvarden (2004), the genus *Ganoderma* has the following salient characteristics: basidiomes annual or perennial, stipitate to sessile; pileus surface with a thick, dull cuticle or shiny and laccate with a thin cuticle or cuticle of clavate end cells; context cream coloured to dark purplish brown, soft and spongy to firm-fibrous; pore surface cream coloured, bruising brown, the pores regular, 4–7 per mm; tube layers single or stratified, pale to purplish brown; stipe when present central or lateral; hyphal system dimitic or trimitic; generative hyphae with clamps; skeletal hyphae hyaline to brown, non-septate, often with long, tapering branches; basidia broadly ellipsoid, tapering abruptly at the base; cystidia absent; basidiospores broadly to narrowly ellipsoid with a truncate apex and apical germ pore, wall two-layered, the endosporium brown and separated from the hyaline exosporium by inter-wall pillars, negative in Melzer's reagent, 7-30 μm long.

As well as basidiospore morphology, features of the pileus context are important for diagnosing species with *Ganoderma;* (1) consistency, (2) structure, and (3) presence of

resinous deposits (Moncalvo and Buchanan 2008; Steyaert 1977; Tham 1998). Only three types of context consistency are recognized. Two — soft-spongy and fibrous — are easy to distinguish, but there is an intermediate state between soft and fibrous that is difficult to identify. The third type was termed "fibrous-spongy." The context structure is classified as (1) duplex, (2) not fully homogeneous, or (3) homogeneous. In the duplex context there is an abrupt change, with two separate contrasting colours, the upper generally light-coloured and the lower generally darker close to the tubes, although shades may vary to some extent. Not fully homogeneous is used when there is an evident colour difference between the upper and lower parts but without abrupt colour changes, while a homogeneous context has only one colour. Contexts with an occasional very thin darker line just above the tubes that is absent in some specimens are considered as homogeneous. The context can contain resin-like deposits (hard and brittle, dull or shiny) that often they form continuous bands or discrete bodies (Torres-Torres and Guzmán-Dávalos 2012).

Other micro- and macro- morphological characters may be variable and cannot be used as reliable characters (Douanla-Meli and Langer 2009; Hong and Jung 2004) for *Ganoderma*. For example some authors (Moncalvo 2000; Patouillard 1889; Seo and Kirk 2000) have dismissed the stipe as a character of no taxonomic value, because some sessile species can develop a stipe in the laboratory resulting from varying oxygen conditions. The presence of chlamydospores is an important characteristic for species identification, as it is also corroborated by molecular phylogenetic studies. Only a few *Ganoderma* species, such as *G. colossus*, *Ganoderma multiplicatum* (Mont.) Pat. and *G. weberianum* contain chlamydospores in their basidiomata (Douanla-Meli and Langer 2009; Hong and Jung 2004).

Chorological data available, through meticulous field work in Europe and other parts of the Northern hemisphere, reveal apparent host affinities that can be of diagnostic value for *Ganoderma* species determination. For instance, several *Ganoderma* species grow on different

angiosperm hardwoods, while others seem to be constantly associated with conifers (Seo and Kirk 2000). In the northern temperate regions *Ganoderma valesiacum* Boud., *Ganoderma carnosum* Pat., *Ganoderma tsugae* Murr. and *Ganoderma oregonense* Murr. have been distinguished from *G. lucidum*, mainly because they are all believed to be restricted to conifers, as discussed above. The host plant, however, cannot always be determined with certainty, since *Ganoderma* basidiomes are often found on heavily decayed wood in mixed forests. However, before conclusions can be reached about host specificity, collections in tropical or subtropical regions still need to be examined so there is a better understanding of species boundaries. This may require new collections as host tree species have often not been reported for many species in tropical countries (Seo and Kirk 2000). Steyaert (1972) was the first to extensively study collections from palms in SE Asia. He reported five laccate and one non-laccate species:

- *G. zonatum*, in America and Africa, mostly on palms but also found on *Eucalyptus*;
- *Ganoderma miniatotinctum*, in South-East Asia and Solomon Islands, found only on palms;
- *G. boninense*, from Sri Lanka to the Pacific islands and Japan to Australia, only on palms;
- *Ganoderma cupreum* (Cooke) Bres., paleotropical, on both palms and woody dicots (since synonymized with *G. mastoporum*);
- *Ganoderma xylonoides* Steyaert, restricted to Africa, on both palms and woody dicots; and *Ganoderma tomatum* (Pers.) Bres., in Asia and some Pacific islands, only on palms.

There has been comparatively little taxonomic confusion about *G. philippii*, the main focus of this thesis. Its pathogenicity to a broad host range has been proven (Agustini *et al.* 2014a; Batista 1982; Lee 2000; Lim 1977) (see sections 1.2. and 2.2.1.).

2.2.3. Aetiology of *Ganoderma* species

Ganoderma root rots spread clonally by means of root contact with infectious fungal material i.e. contact between roots of a healthy host with the substrate it is growing saprophytically on. These include tree stumps remaining from cleared native forest or a

previous rotation, or an infected root from an adjacent tree (Flood *et al.* 2000b; Nandris *et al.* 1987b).

Clonal vegetative infection pathways of oil palm were described for the pathogen *G. boninense* in the early 1930s and remained unchallenged as a principle underpinning management practices for half a century (Sanderson and Pilotti 1997). Primary mycelium (when a haploid germinates) can colonize palm wood but is non-infective (Rees 2006; Rees *et al.* 2007) and many attempts to infect mature palms and seedlings with *G. boninense* basidiospores have not been successful (Hasan *et al.* 2005). Anastomosis with a compatible mating type is required to form the invasive secondary mycelium or heterokaryon (Hasan *et al.* 2005). Germlings readily anastomose (Pilotti 2005) and mating can occur either on the palm surface, or during colonization of organic debris in soil (Flood *et al.* 2000b). Secondary mycelium is similarly critical to the infection pathways in most basidiomycete root rot pathogens e.g. rubber, oil palm, coconut and hardwoods (Ariffin *et al.* 1989; Chang 2002; Flood *et al.* 2000b; Gafur *et al.* 2015; Hushiarian *et al.* 2013; Karthikeyan *et al.* 2007; Nandris *et al.* 1987b; Nelson 1976; Rimbawanto *et al.* 2009). Rees *et al.* (2007) showed that, by attaching infested wood blocks to roots, even a small amount of *G. boninense* inoculum can infect unwounded roots, and progression and invasion follow. *Ganoderma* species, although significant pathogens, often appear poor competitors in non-sterile soils (Cairney 2005; Chang 2003), or in woody organic debris during the saprotrophic component of the life cycle e.g. *G. boninense* (Paterson 2007). This observation has led to the development of biocontrol agents such as *Trichoderma* for the reduction of inoculum potential of debris in the field.

Ganoderma spp. are considered as wet-air spore releasers, because concentrations show marked seasonal differences, with highest numbers during the wet season (Adaskaveg and Gilbertson 1988; Hood 2006). Water is an important factor involved in spore release (Ho and Nawawi 1986a). Lacey (1991) observed an abundance of *Ganoderma* spp. spores during the

wet season in tropical countries. The rainy period accelerates development of sporocarps and the release of more spores because of high humidity and water availability. Oliveira *et al.* (2005) have reported from Portugal that maximum *Ganoderma* spp. spore release occurs during late summer and autumn. The same results were obtained in Melbourne, Australia (Mitakakis and Guest 2001); New Zealand (Kadowaki *et al.* 2010) and in Kerala, India (Jothish and Nayar 2004). Lim (1998) found seasonal trends in 5 of the most common fungal spores collected in Singapore, with peak periods in February-March. Studies to quantify diurnal spore release rates in *G. boninense* have shown that peak numbers are released in Sumatra, Indonesia at 1900 hrs (Rees *et al.* 2011), other studies have found spores of *Ganoderma, Coniophora* and other basidiospores occur in the U.K. and Malaysia at maximum concentrations between 2200 hrs and dawn (Ho and Nawawi 1986a; Sreeramulu 1963).

The relative importance in basidiomycete root rot diseases of new spore infections versus mycelial spread from pre-existing infections is said to be reflected by the size and number of genetic individuals in a stand (Stenlid and Redfern 1998). Many individual mycelia per unit area implies a high level of spore infection (sexual dispersal) while the size of each genet reflects the extent of vegetative (clonal) spread. Knowledge of this balance is important to disease management (see section 2.1.3.). More recent studies suggest that, for *G. boninense*, despite the historic focus given to vegetative infection pathways in disease management, both root-to-root contact and basidiospore dispersal play important roles in disease dissemination (Hood 2006; Miller *et al.* 1999; Soepena *et al.* 2000). This thesis also challenges the role of spores versus secondary mycelium in the dispersal of *G. philippii* and discusses the relevance of this information to disease management (see section 1.7. and 2.6.).

2.3. *Ganoderma* species in Indonesian hardwood plantations

Ganoderma philippii appears primarily responsible for the incidence and spread of red root disease in Indonesian acacia and eucalypt plantations (Coetzee *et al.* 2011). This pathogen has both a broad host range and geographic distribution (Farr and Rossman 2012). It is found in rubber, tea, cocoa and coffee plantations, with reports coming from Brazil, the Congo, Nigeria and Tanzania (Moncalvo and Ryvarden 1997; Steyaert 1977), Malaysia (Lee 1997; Lee 2000), the Philippines (Almonicar 1992; Militante and Manalo 1999), Papua New Guinea (Arentz 1996), India (Mehrotra *et al.* 1996; Prasad and Naik 2002; Sharma and Florence 1997) and Vietnam (Irianto *et al.* 2006). In Indonesia, the disease is widespread (Old *et al.* 2000; Rahayu 1999), but information on the incidence and impact of this root rot is limited to a few studies as commercial companies keep impact data confidential.

Morphological characters of the sporocarps and mycelium in infected roots and phylogenetic analysis (based on DNA sequences) have confirmed that *G. philippii* is the fungal species most commonly associated with red root disease in Indonesian *A. mangium* plantations (Glen *et al.* 2009). *Ganoderma philippii* is distinguished by an especially characteristic double-walled spores with tiny spines or echinulae, positioned between the two wall layers. Spores are ovoid with a truncated apex, pale brownish, 6–9.5 × 4–8 µm. The hyphal system is trimitic with clamped, narrow septate generative hyphae with clamps and wider, thick-walled, non-septate, brown skeletal hyphae that branch near the ends; and narrower, thick-walled, brown, branching binding hyphae. The skeletal hyphae are the most apparent within the tissues, and clamped generative hyphae are not easily seen. Basidiomata are formed of interwoven hyphae, often grouped, imbricate and broadly attached, woody shelf, concentrically furrowed and warty above, smooth, semi-glossy in parts, coloured dark reddish- or purplish-brown, with a narrow, white margin; white or eventually brownish, beneath, with medium-fine pores ((4–)6–7 per mm) (Glen *et al.* 2009; Hood 2006). Infected roots are coated in a reddish or reddish-brown

mycelial crust which has a creamy-white rhizomorph-like growing margin (Mohammed *et al.* 2012).

Agustini *et al.* (2014a) using morphological and molecular identification methods found a high level of red root rot caused by *G. philippii* in *E. pellita* plantations in South Sumatra, even though sporocarps of this species were rarely found. Contrary to expectations from the literature about host pathogen associations, *G. philippii* was almost as prominent as *Phellinus noxius* in causing root rot in *E. pellita* stands (Agustini *et al.* 2014a). *Ganoderma mastoporum* and *G. australe* were also isolated from red root rot affected *A. mangium* and *E. pellita* roots in Sumatra; and were sometimes found co-occurring with *G. philippii* (Yuskianti *et al.* 2014).

Ganoderma mastoporum occurs widely in Asia and Africa (Corner 1983), however it has not previously been found in *A. mangium* outside South Sumatra, and its role in root rot disease needs to be evaluated. In the absence of pathogenicity tests, it is unclear whether *G. mastoporum* can act as a primary pathogen and produce the red mycelial sheath, or is merely a secondary coloniser of damaged roots that have previously been rotted by *G. philippii* (Yuskianti *et al.* 2014). Phylogenetic analysis has however grouped a *G. mastoporum* isolate from sporocarps growing on *A. mangium* in Malaysia (FRIM 98) with South Sumatran collections of this species. *Ganoderma australe* represents a diverse species complex that is putatively widespread across both the northern and southern hemispheres (Moncalvo and Buchanan 2008).

Disease centres associated with *G. philippii* root-rot are easily located in first rotation (1R) *A. mangium* plantations just one year after their establishment; indicating that, like other common tropical root-rot pathogens such as *P. noxius* (Brooks 2002) and *R. microporus* (Nandris *et al.* 1988), *G. philippii* is endemic in the native- or formerly native- forest

environments that are being converted to *A. mangium* monocultures in Indonesia. Disease centres in first rotation sites are thought to arise from inoculum originating from native-forest residual roots colonized by *G. philippii* (Mohammed *et al.* 2014). In Malaysia, incidence of root rot in *A. mangium* tended to be highest in lowland areas of former rainforest due to greater initial inoculum levels (Lee 1997). *Ganoderma* root rot is also absent in *A. crassicarpa* grown in former grassland areas, further indicating a link with previous vegetation and root rot, as forest and grassland vegetation have different fungal compositions (Risna and Suhriman 2003).

Surveys conducted by Irianto *et al.* (2006) in Indonesian plantations recorded the potential for red root rot to kill up to 28% of trees in second rotation *A. mangium* plantations. In Central Sumatra. By the third rotation, several sites were no longer capable of providing a commercial yield at harvest (Francis *et al.* 2014). It was noted that second rotation compartments in Central Sumatra and East Kalimantan province had consistently higher levels of disease incidence compared with South Sumatra indicating the influence of both previous vegetation and climate.

Harvesting and management practices may also influence the incidence of red root rot in *Acacia* plantations. The continuous planting of short rotation crops (combined with a short delay between harvesting and replanting) provides the fungus with a continuous network of stump and root debris to invade and infect (Irianto *et al.* 2006). Lee (2000) noted significant variation in both onset and rates of increase of mortality in *A. mangium* plantations in Malaysia; the worst cases had up to 50% mortality in 10- to 14-year-old plantations, but others had <5% after a similar period and one remained disease free. The plantations had been established on previously logged-over lowland rainforest areas. The harvesting debris had, in most cases, all been mechanically cleared and burnt and the differences in infection level could have reflected the degree of success in the removal of initial inoculum. In Indonesia, harvesting debris is retained and inevitably leads to increased infestation of *G. philippii* via increased sources of root-disease inoculum (Hardiyanto and Wicaksono 2008).

Below-ground symptoms may be readily apparent if soil is excavated around the roots but crown symptoms often do not appear until 50% or more of the root system is destroyed (Mohammed *et al.* 2014). Above-ground symptoms, when observed, include thinning and chlorosis of the crown, reduced leader growth and production of a distress seed crop (Flood *et al.* 2000a; Francis *et al.* 2014). Extensive degradation of the roots of highly susceptible species such as *A. mangium* robs the trees of structural support, making them prone to windthrow. The number and size of gaps become much larger in older and later-rotation plantations, pointing to the rapid build-up of inoculum load from previous crop debris (Francis *et al.* 2014). Disease development is mainly as a result of increases in gap size followed by coalescence where multiple gaps are present. Adjacent mortality clusters could result from neighbouring trees making root contact with the same buried primary or previous crop inoculum (Francis *et al.* 2014). Root-to-root contact is, however, supported by the observation of grafts between excavated acacia roots and visible root infection on both sides of the graft (Lee 2000; Mohammed *et al.* 2012).

2.4. Life cycle, sexuality and population genetics of basidiomycetes with reference to *Ganoderma* species

Relatively little research has been devoted to the life cycle, genetics of sexuality and population genetics of *G. phillipii*. The following discussion is a summary of what is known about the biology and life cycle of basidiomycetes, especially *Ganoderma* species, and what might be inferred for *G. philippii*.

Ganoderma spp. are thought to follow the characteristic life cycle and pleomorphic developmental sequences of the Hymenomycetes (Ariffin *et al.* 1996; Rees 2006). A primary mycelium contains one or more genetically identical haploid nuclei per cell, hence is generally referred to as a monokaryon. A secondary mycelium contains two or more genetically distinct

haploid nuclei in its cells (generally referred to as the dikaryon). The secondary mycelium is typically formed through fusion and nuclear association (plasmogamy) of compatible monokaryons. Plasmogamy occurs at the hyphal tips (tip-to-tip fusion), hyphal tip and a lateral hyphal wall (tip-to-side fusion), hyphal tip and a lateral swelling of the hypha (tip-to-peg fusion), or between lateral swellings of neighbouring hyphae (peg-to-peg fusion Kües *et al.* 2011). Upon fusion, nuclei enter the mycelium of the opposite mating type and, with dissolution of the hyphal septa (Billiard *et al.* 2012), migrate through the hyphae until they reach a hyphal tip cell (Hseu and Wang 1996). Once a migrating nucleus reaches a hyphal tip cell, the two types of haploid nuclei pair and then divide synchronously. Simultaneously, specialized clamp cells are formed at the position where a new septum will appear (Ni *et al.* 2011).

Usually the term heterokaryon is used for a mated mycelium and the term homokaryon for a mycelium arising from a single spore harbouring only one nuclear type (Gladfelter and Berman 2009). The fruiting body is constituted of heterokaryotic mycelial elements. A specialized fertile tissue, the hymenium, forms within the fruiting body and consists of terminal, heterokaryotic club-shaped, spore-bearing cells, the basidia. Within each basidium a heterokaryotic pair of nuclei fuse to form a transient, heterokaryotic nucleus. Meiosis follows to produce four haploid nuclei, which migrate singly into four basidiospores that are formed exogenously upon the basidium (Worrall 1999). The life cycle has been termed haploid-dikaryotic (Raper 1953).

In Hymenomycetes, heterokaryosis is required to complete the life cycle and typically involves combining very different nuclear genomes in the same cell. The heterokaryotic phase in Hymenomycetes is the dominant growth phase in the life cycle and may display indeterminant growth. The vigorous, rapidly propagating heterokaryon is widely accepted as the dominant stage in the *Ganoderma* life cycle. In *G. boninense* anastomosis with a compatible

hypha is necessary for the potentially invasive heterokaryon to form (Pilotti *et al.* 2003). Homokaryotic mycelium derived from a single basidiospore can colonize sterilised oil palm wood blocks (Hasan and Flood 2003) but infection of living palms from a single basidiospore inoculum has never been confirmed in the field.

The existence of a heterokaryotic stage in the life cycle has several evolutionary implications. Heterokaryons are expected to have increased adaptive potential relative to homokaryons due to genetic variation between the two nuclei (Clark and Anderson 2004). Heterokaryons have the potential for additional genetic variation relative to diploidy because the distance between the two nuclei within a cell can vary, which may impact the phenotypic expression (James *et al.* 2008). They may undergo somatic recombination of genomes during vegetative growth, allowing the shuffling of genes between nuclei without the commitment to and complete reassortment of meiosis (Anderson and Kohn 2007; Hansen *et al.* 1993; Pontecorvo *et al.* 1953). Most importantly, although heterokaryons retain the potential for genetic mixture and recombination during vegetative growth, they represent a much less stable union of nuclear genomes relative to diploidy (Anderson and Kohn 2007). When a heterokaryon encounters a homokaryon during vegetative spread, nuclei of the heterokaryon may emigrate into the homokaryotic individual, a process termed as the Buller phenomenon (Raper 1966a; Raper 1966b; Raper 1966c) (See section 2.4.4.). All of these processes of reassortment allow an individual nucleus to interact with other genetically different nuclei without undergoing karyogamy, further suggesting that nuclei within heterokaryons have a degree of autonomy and could be considered distinct units of selection.

2.4.1. Mechanisms governing mating recognition and breeding compatibility in basidiomycetes

The hyphal apex plays an essential role in growth, foraging, nutrient acquisition, sensory recognition and response, and in both somatic and reproductive differentiation (Burnett 2003).

Basidiomycete fungi have elaborate compatibility systems regulating mating, individual integrity, and speciation (British Mycological Society 2008). Their population structure and dynamics are governed by the interplay between the genetically controlled compatibility systems (Kües *et al.* 2011) and the ecological parameters of colonization opportunity (Hood 2006) and substrate continuity (Rayner and Frankland 1984).

Fusions within the same thallus (self-fusions) convert what was a radiate communication system into a network and are also important in binding hyphae together in the plectenchymatous aggregations that form basidiocarps (Billiard *et al.* 2012). Fusions between genetically different thalli (nonself fusions) allow the possibility of genetic exchange, with the eventual outcome dependent on whether or not mechanisms of non-self-rejection (somatic/vegetative incompatibility) or acceptance (sexual compatibility) are brought into operation (Rayner and Frankland 1984). Three known incompatibility systems govern the outcome of these interactions: sexual incompatibility, somatic incompatibility and intersterility (Anderson and Leslie 1992; Billiard *et al.* 2012; Brasier 1987; Rayner 1991).

Several features distinguish somatic incompatibility (SI) from sexual incompatibility and intersterility systems in the basidiomycetes. Essentially, SI is a type of self/non-self-recognition that involves interaction between the somatic tissue of two or more individuals within the same species for which incompatible interactions are associated with some recognition of difference. Like SI, mating compatibility involves interactions between members of the same species. However, mating compatibility systems are involved in the regulation of fusion between gametes or reproductive structures (in plants and ascomycetes) or between primary mycelia (basidiomycetes), while SI regulates fusion between vegetative tissue (ascomycetes) or secondary mycelia (basidiomycetes). Mating incompatibility also differs from SI in that mating incompatibility is associated with genetic similarity, rather than difference. Finally, intersterility interactions are sometimes treated as a type of SI interaction,

since (depending on the species concept employed and the degree of intersterility) they can be considered to involve interactions within species. Like mating compatibility, however, intersterility is associated with contact between reproductive tissue, rather than vegetative tissue or secondary mycelia.

2.4.2. Somatic incompatibility in basidiomycetes

Somatic incompatibility is referred to by Worrall as "the prevention of effective fusion and integration following allorecognition (recognition of non-self) between genetically distinct, conspecific tissues when isogenic (self) contacts result in such fusion. 'Somatic' specifies a non-reproductive domain, distinguished from the system of sexual incompatibility." Somatic incompatibility in fungi can thus be defined as the mycelial rejection between genetically distinct individuals within the same species and is used to maintain individuality of mated or secondary mycelium from genetic exchange. In *Armillaria*, as well as other fungi, when two isolates of secondary mycelium come in contact, they are said to be incompatible if anastomosis fails and a barrage zone (zone of inhibition) forms between them. The isolates are said to be compatible if the two mycelia merge and anastomoses persist between them.

Encounters between dikaryotic individuals from the same incompatibility group generally lead to fusion and may result in the creation of a physiologically connected organism. In contrast, confrontations between genotypes recognizing each other as non-self are followed by a rejection response that prevents or restricts cytoplasmic or nuclear exchange between the two genotypes. In most cases, somatic incompatibility is determined by the nuclear genome, however mitochondrial differences are tolerated, and a mitochondrial mosaic behaves as one individual (May 1988). These processes serve to rapidly separate newly formed and original dikaryons, regardless of any previous history of hyphal fusion; thus ensuring that different dikaryons persist as discrete individuals and continue to function as individual genetic and

physiological units (Ainsworth and Rayner 1986; Malik and Vilgalys 1999; Todd and Rayner 1980).

Mechanisms of vegetative self/non-self-recognition of dikaryons were first suggested from observations of seemingly antagonistic hyphal reactions (mycelial barrages, interaction zones, lines of demarcation) between isolates of the same species from different origins, when paired in axenic culture. These are characterised by pigmented zones, sparse hyphae (clear zones) or tight hyphal knitting, and an increased production of sclerotia at the interface of colonies (Guillaumin *et al.* 1996). Intraspecific SI was recognized in wood-decaying basidiomycetes many years ago and has been used by forest pathologists in most forest-growing regions of the world as a guide to identifying genetically distinct isolates within fungal populations (Bartkute-Norkuniene and Sakalauskas 2011; Brown 1987; Johnson 1972). Discrete mycelia, identified on this basis as being members of one group, have been variously referred to as constituting ‘clones’, 'genets' or having the same 'compatibility genotype' (Leslie 1993). Although little was understood about the physiological basis of the clonal reactions, vegetative incompatibility became regarded as the standard for delineating individuals in a fungal population (Rayner and Todd 1982; Todd and Rayner 1980).

2.4.3. The nuclear cycle: sexuality in basidiomycetes

Basidiomycetes employ systems to regulate the amount of genetic heterogeneity that will be present in the next generation; mating “compatibility” is determined by incompatibility genes known as the mating “type” (Dighton *et al.* 2005). According to the data collected on ~230 species by Whitehouse (1949), ~90% of all basidiomycetes require cell to cell fusion between two independent strains of different mating type for sexual reproduction (heterothallism), whereas ~10% of species are self-fertile and undergo sexual reproduction without a partner of another mating type (primary homothallism Fischer 1994).

For heterothallic mating to be successful, the haploid individuals must be of complementary mating type to undergo cell fusion. Strains of the same mating type are usually incompatible and incapable of forming a dikaryon (Raper 1966c; Whitehouse 1949). Mating in heterothallic basidiomycetes involves the fusion of two self-sterile strains (haploid yeast cells or filamentous monokaryons) each with a distinct type of haploid nucleus. Following cell to cell fusion, nuclear fusion is delayed, and the two nuclei congress and pair but do not fuse, forming a dikaryon with two distinct types of haploid nuclei in its cells, one from each parent (Hiscock and Kues 1999; Meinhardt and Esser 1990). The dikaryon grows as hyphae, in some cases with fused clamp connections, and sexual reproduction occurs, with nuclear fusion (karyogamy), meiosis, and the formation of basidia and basidiospores. The culmination of the sexual cycle is restricted to a single specialized cell, the basidium, which forms at the termini of dikaryotic hyphae (Hood 2006; Kües 2000; Ni *et al.* 2011).

The efficiency of a breeding system is measured in terms of how well it restricts inbreeding and, as a consequence, how much this then imposes a restriction on outbreeding (Burnett 2003). Inbreeding restriction can be maintained without a corresponding reduction in outbreeding potential if there are many different alleles of the mating type genes all of which are cross-compatible (Burdon and Silk 1997). More than 30% of the higher basidiomycete fungi have a "unifactorial" or "bipolar", one gene incompatibility system (Kües *et al.* 2011). However, there are numerous naturally occurring incompatibility alleles for the one gene, and any two individuals are compatible so long as they have different alleles. Thus, the greater number of alleles in nature, the greater the outbreeding potential. Since only two alleles are involved in any mating the meiotic products of bipolar species will always include 50% of each type and the inbreeding therefore remains fixed at 50%, regardless of how many alternate alleles are present in the population (Kües *et al.* 2011). Many such species have been analysed, the number of alleles is always at least 20 (British Mycological Society 2008; Brown and Ogle 1997;

Burnett 2003). Thus the outbreeding potential of bipolar species is at least 95% while the inbreeding stays at 50% (Worrall 1997).

Almost 60% of the higher basidiomycetes are characterised by a more complex "bifactorial" or "tetrapolar" incompatibility system, involving two mating types with many alleles. In this system two factors, A and B, together determine mating competence and two individuals will only be compatible if they have different specifications for each factor. These factors are generally on separate linkage groups and their independent segregation at meiosis leads to the production of four main types of progeny. The inbreeding potential is drastically reduced since any one progeny will be compatible with only 25% of its siblings (Worrall 1997). This reduction of inbreeding per tetrad is compensated for by acquisition of multiple specificities for each of the two mating type loci. Several well-understood multiallelic mating-type systems are present in the tetrapolar basidiomycetes (Casselton and Olesnicky 1998; James *et al.* 2004; Kües *et al.* 2011). Raper (1966a) calculated that there are about 160 and 240 different A and B specificities, resulting in a total of 12,800 – 57,600 different mating types in *Coprinus cinereus*. Even the lower of the two estimates returns an outcrossing probability of 98% and thus inbreeding repression is achieved.

The genetics of sexuality of several G*anoderma* spp. have been determined previously. Tetrapolarity predominates within the genus (Adaskaveg and Gilbertson 1986; Banerjee and Sarkar 1958; De 1980; Triratana and Chaiprasert 1991). The distribution of mating alleles has been used to ascertain the genetic differences and diversity potential of *G. collosum* (De 1980), *G. lucidum, G. fornicatum, G. microsporum* (Hseu and Wang 1996), *G. tsugae,* (Adaskaveg and Gilbertson 1986) and *G. boninense* (Pilotti 2005; Pilotti *et al.* 2002). All were found to possess a tetrapolar type of sexuality with allelomorphs for heterothallism at two loci.

Ganoderma boninense is likewise heterothallic and tetrapolar with multiple alleles at the two mating type loci (Sanderson 2005) encouraging out-crossing, increasing the potential for selection to take place. During the infection process, regardless of how this occurs, selection pressures inevitably lead to build up within the population of genes that code for characters for aggressiveness.

In summary, *Ganoderma* spp. employ the most complex of the known fungal mating systems, with an in-breeding restriction of 25% which favours out-crossing within a population, but this has not been confirmed for *G. philippii*. Such a system requires an active sexual stage to maintain genetic diversity within populations (Pilotti *et al.* 2002).

2.4.4. The Buller phenomenon in basidiomycetes

Following dikaryon formation, there is usually no restriction to hyphal fusion with other monokaryotic or dikaryotic strains, but, in contrast to monokaryons, dikaryons do not accept invading nuclei (Kimura 1982). However, dikaryons are capable of contributing fertilizing nuclei to a haploid monokaryon, resulting in a new dikaryon; this reaction has been termed the Buller phenomenon (Gladfelter and Berman 2009). There are three possible interrelations that may be classified as follows:

(i) COMPATIBLE: both components of the dikaryon are compatible with the monokaryon, $e.g.(A_1B_1 + A_2B_2) \times A_3B_3$

(ii) HEMICOMPATIBLE: only one dikaryotic component is compatible with the monokaryon, $e.g.(A_1B_1 + A_2B_2) \times A_1B_3$

(iii) NONCOMPATIBLE: neither dikaryotic component is compatible with the monokaryon, $e.g.(A_1B_1 + A_2B_2) \times A_1B_2$

In compatible and hemi-compatible combinations, dikaryosis of the monokaryon occurs readily and regularly. However, unlike incompatible monokaryotic combinations, dikaryosis can occur in non-compatible dikaryotic-monokaryotic combinations, but only in isolated portions of the monokaryotic mycelium and usually after some delay. This is made possible as a result of somatic recombination yielding new nuclei of compatible mating type. For example:

$$(A_1B_1 + A_2B_2) \times A_1B_2$$

$$\downarrow$$

$$A_1B_2 + A_2B_1 \rightarrow A_2B_1 + A_1B_2$$

Which means that all dikaryotic-monokaryon combinations have the potential to be sexually fertile (Esser 1962; Esser 1971). There is increasing evidence that the homokaryotic phase in certain cases might be prolonged, especially in protected substrata such as inside the wood of dead or living trees (Garbelotto *et al.* 1999; Kerio *et al.* 2015; Redfern *et al.* 2001; Stenlid 1994), and that such mycelia are able to act physiologically as functional units. From an evolutionary standpoint, homokaryons can be regarded as redundant if they do not find a mate. On the other hand, they can function as facilitators for the establishment of subsequent genotypes in the resource and increase the possibilities for a second spore to establish in their vicinity (Kemp 1975). The domain occupied by a homokaryon is a selective substratum for conspecific spores capable of mating but acts to exclude another competing species. In the phase of early colonization of a resource, this can extend and reinforce the spatiotemporal window for spore germination and increase the chances to establish a fertile heterokaryon (Rayner and Frankland 1984). Potentially, the Buller phenomenon might lead to complicated networks of mating in a resource. In some basidiomycete species, although successfully mated, homokaryotic regions can arise within a heterokaryotic mycelium (Billiard *et al.* 2012). If such regions contact another heterokaryon, a re-mating may occur in line with the Buller phenomenon. Such re-assortment of nuclei in somatic incompatibility interaction zones has

been recorded (in axenic conditions) in *Armillaria heimii* (Abomo-Ndongo *et al.* 2002) and *Heterobasidion annosum* (Hansen *et al.* 1993), and also dense field populations of *H. annosum* (Johannesson and Stenlid 2004). A nucleus, by combining with a large number of mates, can find optimal combinations for its genetic outfit. Selection can then act on the individual nucleus instead of at the heterokaryon level (Ni *et al.* 2011).

2.5. Population genetic studies of basidiomycete root rot pathogens

A population is a collection of actually or potentially interbreeding individuals of the same species living in a given geographic area (Graça 2011). This means that the individual has to be defined and the outer boundaries of the population both in terms of geographic distribution and the potential for interbreeding. Defining the fungal individual has been the topic of several reviews (Hall *et al.* 2010; Malik and Vilgalys 1999; Rayner 1991; Rayner *et al.* 1984). In fungi the problems may lie in the number of genetic constitutions possible and that fungal vegetative mycelia may fragment, so the genetic entity (the genet) becomes composed of several ramets. A population genetics approach has been widely used for solving problems of ecology and epidemiology of fungal pathogens, especially for those basidiomycetes that spread vegetatively in soil (Guillaumin *et al.* 1996).

2.5.1. Somatic incompatibility and mating-type allele testing to identify genetic individuals

The two main methods of identifying genetic individuals traditionally used: (a) somatic incompatibility reactions and (b) mating-type alleles. Somatic incompatibility has been used among *Armillaria* and other fungi to identify clonal isolates (reviewed in Worrall 1997). Using the phenomenon of somatic incompatibility (Korhonen 1978; Stenlid and Redfern 1998), it has been possible to identify genetically distinct secondary mycelia of the same species coexisting within a population. Intraspecific pairings on agar media have been widely applied for the

determination of the structure of fungal populations; this method is still popular because of its simplicity and straightforward results. Mating-type allele tests involve four steps as described by Guillaumin et al. (1996): collection of fruiting bodies, single spore isolation of all mating types, identification of the four mating types from single spore isolates, and pairing of mating types with each other, involving 16 different haploid-haploid matings (Guillaumin *et al.* 1996).

Prior to the development of new identification techniques for species delimitation in the 1970's, annulate *Armillaria* species were characteristically identified as *A. mellea sensu lato* (Gregory *et al.* 1991). Using this broad species concept, *A. mellea s.l.* was characterized by an extraordinarily wide host range, with highly variable virulence and morphology (Gregory *et al.* 1991; Guillaumin and Korhonen 1991). Hintikka (1973) developed a mating test technique based on changes in culture morphology to discriminate species. This technique was utilized by Korhonen (1978) to distinguish five European biological species within *A. mellea sensu lato.*

Single spore isolates of most *Armillaria* species typically produce white and fluffy mycelium with aerial mycelia. If the two single spore isolates are of compatible mating types (conspecific), the mycelium will anastomose and form a crustose flattened morphology typical of diploid mycelium (Hintikka 1973). If the single spore isolates either are incompatible or represent different species, the colonies will not anastomose, and the morphology of each strain will remain white and fluffy. A similar paired mycelium test can be used to identify diploid field isolates obtained from mushrooms, mycelial fans or rhizomorphs. In this type of test, the field isolate is paired with single spore tester isolates of known species. If the paired mycelia are compatible, they anastomose freely and, the morphology of the haploid tester becomes flattened and crustose, indicating that the diploid field isolate is of the same species as the single spore tester isolate (Guillaumin and Korhonen 1991; Rizzo *et al.* 1995).

Important root rot basidiomycetes have been studied using either one or both of the methods (Abomo-Ndongo *et al.* 1997; Anderson and Ullrich 1979; Chase and Ullrich 1983; De 1980; Fischer 1994; Gioia *et al.* 2003; Guler 2008; Hseu and Wang 1996; Miller 1995; Pilotti 2001; Pilotti *et al.* 2002; Triratana and Chaiprasert 1991). Such studies have revealed much information the population structure and dynamics, permitting inferences to be drawn about disease epidemiology and management e.g. *Phaeolus schweinitzii,* (Rayner and Todd 1982; Todd and Rayner 1980), *H. annosum* (Korhonen and Stenlid 1998; Stenlid 2008b), *Armillaria* spp. (Anders 1992; Anderson and Kohn 1995; Prospero *et al.* 2008; Prospero *et al.* 2003), *Collybia fusipes* (Marcais *et al.* 2000; Marcais *et al.* 1998), *Rigidoporus* and *Ganoderma* spp. (Adaskaveg and Gilbertson 1987; Chase and Ullrich 1983; Latiffah and Ho 2005; Lim *et al.* 2008; Miller *et al.* 1999; Pilotti *et al.* 2003; Suwandi *et al.* 2004).

The main criticism of SI is that the degree of resolution in distinguishing genets depends upon the number of genetic loci determining this phenotype, the degree of polymorphism at these loci, and the pattern of mating in the population. This critical information affects the probability that two genets will have the same SI phenotype by chance inheritance of the same determinants, and hence the degree of confidence in the accuracy of SI tests in distinguishing genets. For most populations of basidiomycetes, this information is not available. In many cases, somatic incompatibility groups might include several genets which can be of independent origin e.g. for the heterobasidiomycete *Rhizoctonia solani* (Vilgalys and Cubeta 1994), somatic compatibility groups encompass isolates that are different from one another in other genetic characteristics. In the homobasidiomycetes, however, somatic incompatibility testing appears more closely to resolve individual genets (Esser 1971). This might be owing in part to the fact that in this group of fungi, the SI response being tested is between dikaryotic or diploid mycelia (which arise from the mating of two haploids), in which two nuclei contribute to the somatic compatibility (Esser 1971). However, the genetic bases of somatic

incompatibility in basidiomycetes are less well known compared to species of ascomycetes such as *Leurospora crassa, Podospora amerina, Ophiostoma ulmi, Cryphonectria parasitica, Aspergillus* spp. (Worrall 1997).

Interpretation of morphological methods to investigate species and genetic diversity often requires substantial experience (Panchal and Bridge 2005). Further, the time and labour required for morphological species determination can be prohibitive (Panchal and Bridge 2005). Molecular methods for identification and genetic determination have been found to be much more efficient. They are not influenced by effects of different environmental factors, physiological stage of an individual and they are not tissue specific (Glen 2006b; Putman and Carbone 2014; Utomo *et al.* 2005; Zakaria *et al.* 2009).

2.5.2. Molecular approaches to identify genetic individuals and studying populations

During the selection of appropriate methods, several factors should be considered such as the type of sample that is available, the levels of systematics that is being investigated, cost of the process, ease of the technique, development time, inheritance pattern and the source of DNA sample (Govindaraj *et al.* 2015).

A population is regarded as genetically diverse if a substantial proportion of the genes are polymorphic. A polymorphic gene is one for which the most common allele has a frequency of less than 0.95 (Mercière *et al.* 2015). Smith et. al. (1990) were among the first to use molecular markers to compare fungal genets (clones) in the field. They compared mating-type allele tests to mitochondrial DNA restriction fragment patterns and found good correspondence between the two methods, even though mating types are encoded by nuclear DNA. Subsequent studies determined that using DNA fingerprinting or analysis of nuclear DNA tend to correspond much better to somatic incompatibility reactions and mating-type allele tests

(Casselton and Olesnicky 1998; Gatto *et al.* 2009; Korhonen *et al.* 1998; Ota *et al.* 2000; Young-Jin 2012; Zakaria *et al.* 2009).

Many molecular methods can detect naturally occurring polymorphism in fungal DNA (Shu-Jing *et al.* 2006). Some of these methods do not rely Polymerase Chain Reaction (PCR), while some are PCR based methods. The two common non-PCR methods are DNA fingerprinting and Restriction Fragment Length Polymorphism (RFLP). The PCR based methods include: Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), microsatellite analysis, DNA sequencing, Single Nucleotide Polymorphism (SNP), Single Strand Conformational Polymorphism (SSCP) and Sequence Related Amplified Polymorphism (SRAP) (Shu-Jing *et al.* 2006; Tsui *et al.* 2011; Zakaria *et al.* 2009).

Microsatellites and SNPs have become the most popular molecular markers for describing neutral genetic variation in populations of a wide range of organisms (Tsykun *et al.* 2017). Microsatellites, also known as simple sequence repeats (SSRs) are short repeats of DNA nucleotides usually 20-60 bp in length with tandem repetition of 2-5 base pairs such as (GT)n, (CA)n, (CAA)n, or (GACA)n (Field and Wills 1996). These repeats are a common feature in all eukaryotes (occurring in as many as 10^5 different chromosomal loci). With such a wide distribution in eukaryotic genomes, co-dominance (i.e. in diploid organisms, the alleles on both chromosomes are revealed), assumed neutrality, high levels of length polymorphism, high levels of accuracy and reproducibility, and relative ease of use, profiling for microsatellite variability provides a simple and reliable method to identify individual genotypes for studies in population genetics especially in basidiomycete population genetics (Jarne and Lagoda 1996). The advent of next generation sequencing techniques has considerably accelerated and simplified detection of microsatellite loci (Gardner *et al.* 2011).

A single-nucleotide polymorphism marker is based on substitution of a single nucleotide at a specific position in the genome, where each variation is present to some appreciable degree within a population. SNPs occur twice as frequently in intergenic and non-coding regions of the genome than in coding regions (Mu *et al.* 2011). However, SNPs located in non-coding regions are often physically linked to functional or regulatory genomic sites, thus reflecting, for example, selection signatures (Brodie *et al.* 2016). SNPs are mostly bi-allelic and traditional population genetic statistics can easily be applied to them but a higher number of sufficiently polymorphic loci might be necessary to reach the same power as multiallelic SSR loci (Guichoux *et al.* 2011). Detection and genotyping of SNPs has been facilitated by next generation sequencing techniques, and existing DNA databases may also be mined for informative loci in well-characterised genera such as *Armillaria* (Dutech *et al.* 2017).

Because of the intense debate surrounding the validity of using either of these methods and the increasing popularity of SNPs, Tsykun *et al.* (2017) compared the information from both methods, evaluating the utility of SSRs and SNPs for the neutral genetic structure of *Armillaria cepistipes* (basidiomycetes) at different spatial scales. The two markers showed different patterns of structure within the two spatial scales studied. The multiallelic SSR markers seemed to be best suited for detecting genetic structure in indigenous fungal populations at a rather small spatial scale (radius of ~ 50–100 km). In contrast, the pattern observed in SNP markers reflected ancient divergence of distant (~1000 km) populations that, in addition, were separated by mountain ranges.

Population genetics studies using microsatellite markers have proved informative for the management of root rot pathogenic fungi distributed in cool- or warm-temperate areas, including *Armillaria* spp. (Baumgartner *et al.* 2009; Prospero *et al.* 2010; Worrall *et al.* 2004), *Heterobasidion* spp. (Garbelotto *et al.* 2013; Oliva *et al.* 2011) and *Ganoderma* species

(Ekandjo and Percy 2012; Mercière *et al.* 2017; Mercière *et al.* 2015; Nudin and S 2012; Qian *et al.* 2013).

2.6. Research priorities for the management of *Ganoderma* species in Indonesia acacia plantations

Indonesian commercial companies growing *A. mangium* have suffered large economic losses due to both root rot disease and *Ceratocystis* wilt and canker. This has led to a shift away from growing this species to growing *E. pellita* which appears less susceptible than *A. mangium* to these fungal diseases. *Eucalyptus pellita* has been planted on sites that are known to be high risk for root rot or that have had a history of high levels of root rot when planted to *A. mangium*. However, Agustini *et al*. (2014a) reported increasing levels of red root rot in *E. pellita* plantations and positively linked tree deaths to *G. philippii*, indicating that second-rotation *E. pellita* may suffer a much higher incidence of root rot than in the first rotation (Agustini 2009). In perennial crops, infections of woody tissues by root rot pathogens have the opportunity to slowly develop and expand as conditions permit. Infective material can remain viable in the ground for many months and infect subsequent crops at replanting (Eyles *et al.* 2008; Nandris *et al.* 1987b). It is therefore very important to manage in such a way as to minimize the risks to both existing and future plantings from a build-up of inoculum.

Initial disease management practices in the Indonesian pulpwood industry have consisted of ad-hoc testing of the application of treatments known to reduce the inoculum potential in other types of basidiomycete root rot diseases associated with both temperate (Stenlid and Redfern 1998) and tropical forestry (Eyles *et al.* 2008; Nandris *et al.* 1987b). These include silvicultural (planting techniques, root excision and isolation trenching, stump removal and thinning Cleary *et al.* 2013; Pratt 1998; Rimbawanto *et al.* 2009), chemical (fungicide drenching, stump urea Eyles *et al.* 2008; Krokene *et al.* 2008; Soepena *et al.* 2000) and biological (fungal antagonism

or endophytic protection Pratt *et al.* 1999; Rayner and Todd 1977; Sariah 2003; Sariah and Zakaria 2000; Sundram *et al.* 2008; Susanto *et al.* 2005; Thor 2001; Zhan *et al.* 2015). This entirely pragmatic approach has met with limited success in terms of cost effective reduction of root rot incidence (Mohammed *et al.* 2012). Although industrial growers of *A. mangium* can learn from accumulated knowledge and experiences of root-rots in other forest systems, direct transference of management strategies from one system to another without an understanding of host/pathogen biology risks being based on incorrect assumptions.

Current research into management strategies are focusing on genetic resistance and bio-control approaches. They are also the strategies more likely to be deployed with success over the many thousands of hectares involved in tropical *Acacia* plantations. However, screening of genetic trials in the field and artificial inoculation in pot experiments with *A. mangium, Acacia auriculiformis* Cunn. ex Benth., and *Acacia crassicarpa* Cunn. ex Benth., have not yet shown any exploitable trends in resistance to root-rot disease (Mohammed *et al.* 2014). The bio-control research has focused on the development of strains of fungi that show antagonistic activity against root fungal pathogens, or with fungi that colonise and protect roots endophytically e.g. species of *Trichoderma*, *Gliocladium*, *Cerrena* and *Phlebiopsis* (Hill *et al.* 2010; Mohammed *et al.* 2012; Prasad and Naik 2002; Susanto *et al.* 2005; Widyastuti 2006). To date, several are showing promise in *Acacia*; however, effective control of *Ganoderma* root rot in the field with either group of species when applied to the growing medium has been incomplete (Mohammed *et al.* 2014).

Effective reduction of root rot incidence has been achieved in tropical tree crops such as oil palm in South-East Asia (Ariffin *et al.* 2000) and rubber in Malaysia and Africa (Nandris *et al.* 1987a; Nandris *et al.* 1987b). Although these crops have longer histories of disease management than *Acacia*, and carry higher value to support the costs of disease interventions, disease reduction has only been achieved by gaining a thorough understanding of the biology

and aetiology of the pathogens involved in causing disease (Chee 1990; Cooper *et al.* 2011; Sariah 2003). Disease progress curves, not indicative of secondary root spread, have moved researchers to question the role of vegetative transfer of basal stem rots and further investigate population structures of *G. boninense* on oil palm. On the basis that the number and size of genetic individuals within a population reflect the relative importance of spore infections versus vegetative spread (Liyanage *et al.* 1980; Nordén and Larsson 2000; Rishbeth 1988; Sanderson *et al.* 2000) high levels of genetic diversity found in oil palm populations strongly implicated basidiospores in disease progression (Ariffin *et al.* 1996; Miller 1995; Pilotti 2005; Pilotti *et al.* 2003). It is now known that basidiospores are the primary means of *G. boninense* spread in oil palm: airborne basidiospores infect directly via cut frond wounds and indirectly through roots via colonized debris. New infections from mycelial contacts do occur, but far less frequently than was previously thought (Rees *et al.* 2011).

This has had far-reaching management implications for the growers of oil palm, particularly at replanting, not only because the brackets on old infected palms are generally located near to young plants, but also because populations of spores have arisen from an established generation of *Ganoderma* that has already been through rotation cycles. Control strategies are currently being developed based on maintaining zero incidence of *Ganoderma* basidiomes within plantations, especially during the replanting cycle, thus breaking the disease cycle and preventing the infection process (Sanderson and Pilotti 1997).

Ganoderma species are notoriously variable and difficult to characterize and this has led to much past confusion in disease aetiology and epidemiology (Flood *et al.* 2000b). Research has been greatly enhanced through the development and use of molecular markers to discriminate among pathogen populations and individuals (Flood *et al.* 2000b). These technological tools can form powerful adjuncts to field observation and experiments in understanding mechanisms of disease spread and pathogen survival (Flood *et al.* 2000b), providing new insight into disease

epidemiology that enables the implementation of appropriate and effective management strategies. Little research has been done regarding the genetic systems and population genetics of *G. philippii*. Most of the work completed to date is concerned with vegetative incompatibility reactions of paired heterokaryons (Mohammed *et al.* 2012) which were difficult to interpret. This research indicated that population diversity may increase with rotation number meaning that spores gave rise to new disease foci (Mohammed *et al.* 2014). Sporocarps of *Ganoderma* species, especially *G. philippii* are sporadically observed in the short rotation span of *A. mangium* although can be abundant at the end of a 6-7-year rotation (Mohammed pers. com.). Many sporocarps were found growing on dead 10-year-old *A. mangium* trees in a plantation at Bidor, Perak (Núñez and Ryvarden 2000).

Increased knowledge of the reproductive biology, population structure, genetic variation and gene flow of *G. philippii* is therefore required to underpin effective disease management (Rimbawanto *et al.* 2009). We suggest following a similar strategy and techniques employed for *G. boninense*. Studies attempted to improve the understanding of the biology and diversity of *G. boninense* by characterising its modes of sexual reproduction (Pilotti *et al.* 2002) and its mating type systems (Pilotti *et al.* 2003). Trials to assess the relative importance of remnant stumps, woody debris and roots from the previous rotation as potential sources of inoculum at replanting, molecular fingerprinting of genetic variation were used to confirm the origin of the pathogen in infected oil palm seedlings (Flood *et al.* 2000b). The genetic diversity of *G. boninense* has recently been investigated through the development and application of microsatellites (Mercière *et al.* 2017; Mercière *et al.* 2015; Miller *et al.* 1999). Mercière (2017) brought new insights into how the pathogen spreads both short and long distances. Similar information for *G. philippii* will provide a better basis for control options such as stump treatments, the deployment of biocontrol and the overall aim of reducing inoculum levels.

CHAPTER 3. *GANODERMA* BASIDIOSPORE GERMINATION RESPONSES AS AFFECTED BY SPORE DENSITY, TEMPERATURE AND NUTRIENT MEDIA

Abstract

The present study investigated the axenic basidiospore germination of *G. australe*, *G. mastoporum* and *G. philippii* at three spore densities in five nutrient media encompassing a range of carbohydrate complexity, in combination with sawdust and/or ethanol as medium additives. Five incubation temperatures ranging from 10–35°C were used, totalling 300 treatment combinations. A Weibull-type nonlinear regression model, with the asymptote occurring at 50–75 h, gave good fits to the observed data on percentage spore germination at each treatment combination for all three *Ganoderma* species. *Ganoderma australe* and *G. mastoporum* basidiospores germinated on all media, whereas *G. philippii* basidiospores required media that contained 2% ethanol. This study has successfully demonstrated a simple method for the collection and germination of *G. australe, G. mastoporum* and *G. philippii* basidiospores. The best medium for *G. australe* and *G. mastoporum* basidiospore germination was rice dextrose agar with a mixture of *Eucalyptus* and *Acacia* sawdust, whereas for *G. philippii* it was 1% malt extract agar plus ethanol, with or without sawdust. Spore density was also critical to achieving the best germination rate, with ~400 spores/cm^2 optimal for all three species. As *Ganoderma* root rot disease affects commercial *Acacia mangium* and *Eucalyptus pellita* plantations, the greater understanding of basidiospore germination gained from the current study should assist in developing strategies to contain the dispersal and spread of root rot in Indonesia and other southeast Asian countries where these species have been planted.

3.1. Introduction

Ganoderma spp. are frequently associated with root rots of tropical *Acacia* and *Eucalyptus* plantations, and *Ganoderma philippii* has been identified as the dominant pathogen causing red root rot disease in both *Acacia mangium* and *Eucalyptus pellita* plantations in Indonesia and Malaysia (Agustini *et al.* 2014a; Coetzee *et al.* 2011; Mohammed *et al.* 2012; Yuskianti *et al.* 2014). *Ganoderma australe* and *Ganoderma mastoporum* are also associated with *A. mangium* plantations in Sumatra (Glen *et al.* 2009). *Ganoderma mastoporum* produces abundant sporocarps and is occasionally isolated from *A. mangium* roots (Yuskianti *et al.* 2014), but in the absence of pathogenicity tests it is unclear whether this species can act as a primary pathogen or is merely a secondary coloniser of damaged roots. *Ganoderma australe* represents a diverse species complex that is globally widespread (Moncalvo and Buchanan 2008). It is primarily known as a decay agent of dead wood.

Knowledge of the biology and ecology of these three *Ganoderma* species is limited compared with *Ganoderma boninense*, an important pathogen of oil palm that causes basal stem rot (Cooper *et al.* 2011; Flood *et al.* 2000b). The genetic diversity in *G. philippii* populations is also poorly understood. Disease incidence surveys indicate that vegetative transmission may occur (Francis *et al.* 2014); however, the role of basidiospores in transmission is yet to be established. Previous studies have demonstrated difficulties in obtaining germination of *G. philippii* basidiospores *in vitro* (Lim 1977). For *Ganoderma* spp. in general, mature basidiospores are not easy to obtain from detached polypore sporocarps, but can be collected *in situ* during the fruiting season (Hilton 1961; Kadowaki *et al.* 2010). Maximum spore release occurs overnight for *G. boninense* (Ho and Nawawi 1986a; Rees *et al.* 2011) and *G. applanatum* (Sreeramulu 1959; Sreeramulu 1963). Compared with the oil palm pathogen *G. boninense* (Ho and Nawawi 1986b) and the commercially valuable *G. lucidum* (Karadeniz *et al.* 2013; Liu *et al.* 2001; Magday *et al.* 2014; Srivastava *et al.* 2010), relatively

few publications describe investigations into the factors that influence basidiospore germination in the three *Ganoderma* species in this study (e.g. Lim 1977 attempted germination of *G. philippii* basidiospores but no studies into germination of *G. mastoporum* or *G. australe* basidiospores were found). Experimental confirmation of the conditions required to break dormancy in *Ganoderma* spores have been hampered by the difficulty in demonstrating the ability of spores to germinate *in vitro* (Adaskaveg and Gilbertson 1986; Bazzalo and Wright 1982; Brown and Merrill 1973). However, manipulation of nutritional and environmental conditions, sometimes combined with treatments such as grinding, enzymolysis or passage through insect guts have successfully induced axenic germinations of *G. boninense* (Ho and Nawawi 1986b), *G. lucidum* (Liu *et al.* 2001), and *G. philippii* (Lim 1977) basidiospores.

Exogenous nutrients absorbed from the environment also affect spore germination, thus helping to define their ecological niches. Germinating fungal spores utilise organic sources of carbon with varying degrees of success among species (Gao *et al.* 2007). Niederpruem and Dennen (1966) reported variable rates of axenic germination in *Schizophyllum commune* Fr. when provided with soluble simple carbohydrates. The carbohydrate sources that stimulated earliest germination (between 15 and 20 h) included glucose, xylose, fructose, mannose, turanose, cellobiose, sucrose and galactose. Ho and Nawawi (1986b) determined that the incidence of germination of *G. boninense* basidiospores was greater on complex media such as lima bean agar and led to rapid and more profuse mycelial development compared with germination on defined media.

The ability of basidiospores to germinate depends largely on the rehydration and activity of the intracellular water in spores. As basidiospores have a low water content, poor germination levels are generally observed at low to medium relative humidity (RH), and some species will only germinate at high RH or in the presence of free water (Dong *et al.* 2006; Kuhlman and Pepper 1994). Water uptake is an active process and requires a change in permeability of the

spore wall (Fries 1984). Srivastava et al. (2010) recorded a substantial increase in germination of *G. lucidum* spores provided with a 2% ethanol media additive, and suggested that this was associated with chemical degradation of the sporoderm. Increased axenic germination has been recorded in *G. philippii* (Lim 1977) and *G. lucidum* (Srivastava *et al.* 2010) basidiospores when wood was present as a media substrate.

This study details a simple and efficient method for the collection and axenic spore germination of basidiospores of *G. australe*, *G. mastoporum* and *G. philippii* under different nutritional and physical conditions. A Weibull-type model is used to describe the germination dynamics and measure the effects of spore density, soluble simple and complex carbohydrates, the presence of ethanol and woody substrates, and incubation temperature on the course of germination.

3.2. Materials and methods

3.2.1. Spore collection

Spores were collected opportunistically and non-destructively from basidiocarps at three locations: Langgam (0º07′48″N/101º36′0″E), two *G. philippii* basidiocarps from separate stumps ca. 1 km apart in a *E. pellita* stand; Logas (0º17′24″S/101º16′12″E), one *G. mastoporum* basidiocarp growing in a mature-age stand of *A. mangium*; Baserah (0º12′36″S/101º28′48″E), two *G. australe* basidiocarps from two mature-age *A. mangium* wildling trees, approximately 750 m apart in a mature-age stand. Basidiocarps were tentatively identified according to morphological characters as previously described (Glen *et al.* 2009).

A sterilised 50-mm diameter Whatman filter paper disk was pinned just below the hymenial surface of each basidiocarp, 5–10 mm below the pores (Rees *et al.* 2011). To minimise airborne contamination, the entire basidiocarp and collection apparatus were wrapped in aluminium foil. Collection occurred overnight between 1800h and 0800h. Collection disks were unpinned from the basidiocarp and individually stored for transport face up in sterile 50 mm Petri dishes. The

basidiocarps were removed from the tree, placed in paper bags, dried for two weeks and then vacuum sealed for storage (Herbarium Accession Nos. BO22945-BO22949). DNA was extracted from each parent basidiocarp and species confirmed using species-specific PCR tests for *G. philippii* and *G. mastoporum* (Yuskianti *et al.* 2014) and PCR and rDNA ITS sequencing for *G. australe* (Glen *et al.* 2014). The sequences for the *G. australe* collections and isolates used in this study had over 99% sequence similarity (2 mismatches over 660 bp) to KJ654369.1 and many other accessions of *G. australe*, with the next best match being 94% similarity to KY708881.1, *G. meredithiae.*

Filter papers were air-dried for 15 min, and then stored in sealed dark containers at room temperature. Spores were suspended by placing a section of the spore collection disc in 10 mL of sterile distilled water (SDW) and mixing thoroughly using a vortex. The spore suspension was incubated in the dark for 4 h at 27ºC in a solution containing, per litre, 50 mg penicillin, 50 mg streptomycin, 25 mg polymixin, 1 mL thiabendazole solution (23 mg mL^{-1} in lactic acid). Spores were then collected onto sterile 5 µm filter paper using a perforated ceramic funnel filter, and re-suspended in 50 mL SDW.

3.2.2. Experimental design

A full factorial experiment to investigate the effect of the four factors resulted in 300 treatment combinations for each species (three spore densities × five nutrient media × four medium additive combinations × five incubation temperatures), as follows: Spore density (1×10^4, 3×10^4, 8×10^4 spores/mL in SDW); Nutrient media of high and low concentrations of simple and complex soluble carbohydrates (five different base media, see Table S3.1); Media additives (the presence/absence of 2% ethanol and 10 g sawdust in all four combinations, NIL=neither present, SAW=sawdust only, ETH=ethanol only, SAW & ETH= both sawdust and ethanol, the sawdust prepared from a mixture of ground-up *Eucalyptus* and *Acacia dealbata* Link wood and roots); Incubation temperature (10, 22, 27, 31, 35°C). Each of

the 300 treatment combinations was replicated 4 times (twice in each of two runs). All replicates were prepared using the same spore suspensions. Plates were equilibrated to their respective incubation temperatures for 24 h prior to inoculation.

3.2.3. Collection of germination data

Germination data were collected directly after inoculation (t = 0 h), to ensure no germination had occurred during preparation, and every 24 h for a period of up to and including 4 d (t = 96 h). Germination was measured by counting 300 basidiospores in a random transect over each Petri dish under 10× ocular/50× phase contrast magnification using a Zeiss Axioskop. Spores were counted as germinated when the germ tube reached a length equal to the shorter diameter of the germinating spore. For each observation time, a cumulative germination percentage ($G_{\%}$) was recorded. This was also converted to average germinated spores per cm^2 by multiplying $G_{\%}$ by the expected number of spores applied to each plate (2,500, 7,500 or 20,000) and dividing by the surface area of the plate (19.6 cm^2).

3.2.4. Statistical modelling

All statistical modelling and tests were undertaken using the SAS System for Windows V. 9.2, SAS Institute Inc., Cary, NC, USA. Preliminary trials with nonlinear regression models including the logistic, Gompertz and Weibull-type models, resulted in the Weibull-type model ranking first. This takes the form:

$$G_{\%}(t) = M\left(1 - \mathrm{e}^{\left\{-\left(\frac{t}{b}\right)^{c}\right\}}\right) \text{(Equation 1.1)}$$

where $G_{\%}(t)$ is the cumulative percentage of germinated spores at time t, M is the maximum cumulative percentage germination, b is the time taken in hours for $G_{\%}$ to reach 63.2% of the maximum M, and c is a dimensionless shape parameter. The higher the value of c (holding the other parameters constant), the greater the slope of the response curve in the region where t=b. Other workers, for example Koide and Yasokawa (2008), used exactly the same model to

describe the growth of the mycelial mat and fruiting zone diameters of *Aspergillus niger* Tiegh. subjected to temperature changes. Equation 1.1 can be made more general using an additional time parameter t_0 which estimates the lag phase, i.e. the time to germination onset (Brown 1987),

$$G_{\%}(t) = M\left(1 - \mathrm{e}^{\left\{-\left(\frac{t-t_0}{b}\right)^c\right\}}\right) \qquad \text{(Equation 1.2)}$$

This extended form of the model was used in other studies, e.g. by Wu et al. (1999) to model the effects of temperature and wetness duration on the infection of peanut cultivars by *Cercospora arachidicola* Hori, and by Furuya et al. (2009) to model spring onion infection by *Puccinia allii* (DC.) F. Rudolphi urediniospores, but as the additional time parameter did not substantially improve the fit for the data in the present study, the simpler Equation 1.1 was used to model the cumulative percentage of germinated spores for the 300 treatment combinations. Model fitting was carried out using the procedure PROC NLIN of SAS V. 9.2, with parameter estimates calculated using ordinary least squares estimated by the Gauss iterative algorithm as described by Seber and Wild (2005). The goodness-of-fit of Equation 1.1 to the data for cumulative percentage of germinated spores at each treatment combination was assessed using root mean squared error (RMSE), a measure of the average deviation between the observed data and the fitted model.

Modelling the cumulative percentage of germinated spores at each of the 300 combinations of the four design factors and the three species produced estimates of the parameters *M*, *b* and *c* for each treatment combination. These answer such questions as which treatment combination achieves the highest percentage germination (via the asymptote *M*), what is the time required (via the parameter *b*) to reach 63.2% of that asymptote, and what is the overall shape (via the parameter *c*) of the sigmoidal response curve. Analysis of variance (ANOVA) of the individual estimates of *M* was carried out using the procedure PROC GLM.

3.3. Results

3.3.1. Modelling percentage germination

Not all of the 300 treatment combinations resulted in successful germination within 96 h, with most of the failures involving spore densities of 1×10^4 spores/mL and 8×10^4 spores/mL for each of the *Ganoderma* species. At these spore densities, significantly lower percentage germination was recorded (Table 3.1). At the lower concentration, an increased time to the onset of germination was also observed, whereas, at the higher spore concentration, *G. australe* and *G. mastoporum* commenced germination within 24 h, in contrast to *G. philippii,* which did not show any signs of germination until after 48 h. Graphs from the fitting of Equation 1.1 to the data at the most successful spore density of 3×10^4 spores/mL are shown in Figures 3.1, 3.2 and 3.3. We examine each species separately.

Table 3.1. Average cumulative germination rates and average spores per cm2 of *Ganoderma australe*, *G. mastoporum* and *G. philippii* basidiospores, plated at concentrations of 1 × 104, 3 × 104 and 8 × 104 spores mL- 1 over 96 hours

Species	Spore conc. (× 10^4 mL^{-1})[a]	Cumulative % germination (SE) at time t hours[b]					Average spores per cm^2
		0	24 h	48 h	72 h	96 h	
Ganoderma australe	1	0	0	0	10.6 (0.48)	10.5 (0.62)	127
	3	0	0.7 (0.21)	21.7 (1.82)	26.6 (1.74)	26.1 (1.55)	382
	8	0	2.9 (0.85)	7.7 (0.64)	10.6 (0.69)	11.8 (0.70)	1019
Ganoderma mastoporum	1	0	0	11.2 (0.76)	11.5 (0.71)	14.3 (0.86)	127
	3	0	17.6 (1.76)	24.7 (1.68)	27.7 (1.71)	29.7 (1.79)	382
	8	0	10.7 (1.07)	11.3 (0.77)	12.6 (0.78)	13.5 (0.82)	1019
Ganoderma philippii	1	0	0	0	0.1 (0.03)	7.8 (1.51)	127
	3	0	0	7.0 (1.30)	8.5 (1.48)	8.6 (1.49)	382
	8	0	0	0	0.3 (0.05)	2.0 (0.41)	1019

[a]Entries for each spore concentration for a given observation time for each species are calculated as the mean of the average of the four replicates of the 60 treatment combinations across two experimental runs of nutrient medium (DWA, LBA, MA1, PDA, RDA), media additive (ETH, NIL, SAW, SAW & ETH) and three temperatures (22, 27, 31°C). [b]SE (standard error of the mean) in parentheses.

Ganoderma australe

Maximum percentage germination of *G. australe* basidiospores occurred on Rice Dextrose Agar (RDA) combined with sawdust additive at a temperature of 22°C, with 27°C a close second (Figure 3.1). In all combinations, either 22 or 27°C was generally the best temperature, with 31°C occasionally the second best. Cumulative germination was generally poor at 10°C and also at 35°C. At all temperatures, the shape of the cumulative germination curve was generally sigmoidal, the curve having a positive slope for times up to ca. 40–50 h, where an inflection point usually occurred followed by a levelling off (Figure 3.1).

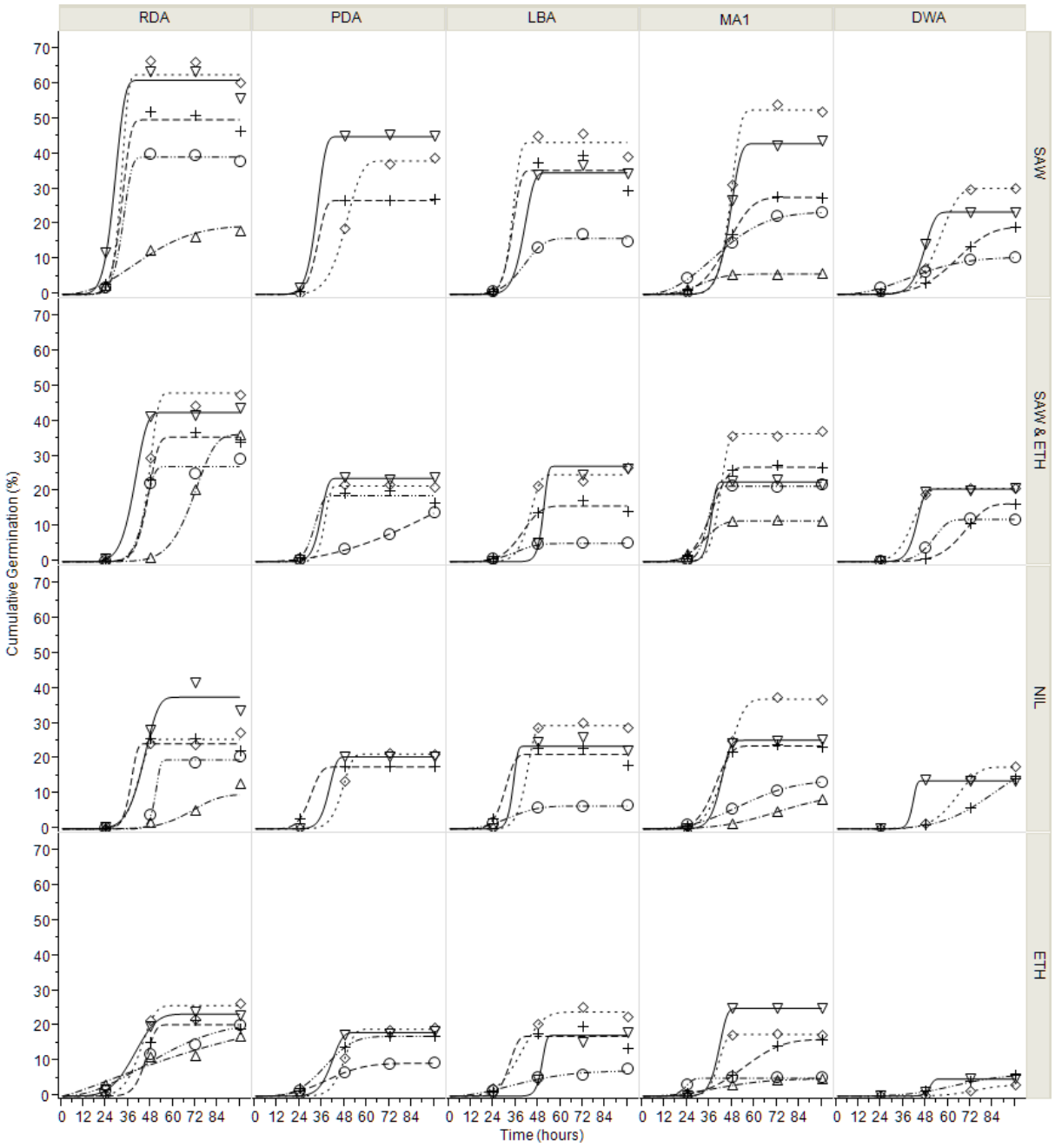

Figure 3.1. Cumulative percentage germination of *Ganoderma australe* basidiospores, plated at 3 × 104 spores mL-1, over a 96 hour period, fitted by the Weibull-type model, Equation 1.1. Effects of five different nutrient media: Rice Dextrose Agar (RDA); Potato Dextrose Agar (PDA); Lima Bean Agar (LBA); 1% Malt Extract Agar (MA1); Distilled Water Agar (DWA) from left to right, in approximate order of decreasing germination. The effect of the additive: sawdust (SAW), 2% ethanol (ETH), both sawdust and ethanol (SAW & ETH) or none (NIL), is presented, from top to bottom, in combination with the medium in approximate order of decreasing germination success. Points represent observed data at the five different incubation temperatures △ 10 ◇ 22 ▽ 27 + 31 ○ 35, as the means of four replicates across two experimental runs. Error bars are not shown for visual clarity. Curves represent fitted models at five constant incubation temperatures signified by 10 22 27 31 35; see Table S3.2 for parameter estimates and goodness of fit. Missing data points represent treatment combinations where no germination was recorded and hence, the data could not be modelled.

Ganoderma mastoporum

The results for *G. mastoporum* (Figure 3.2) had some similarities to those for *G. australe*, but also some noticeable differences. Successful germination was not achieved at either 10°C or 35°C, the optimum temperature almost always being 27°C. A clearly perceptible difference between the shapes of the curves in Figure 3.2 compared with those in Figure 3.1 are the very steep slopes at 24 h or less, the percentage germination almost always reaching close to the maximum by 48 h. As with *G. australe*, the single best treatment combination was RDA with sawdust additive. The addition of alcohol had either a small positive (e.g. on DWA) or no effect. This contrasts with *G. australe* (Figure 3.1), where the addition of ethanol, with or without sawdust, tended to have a small, but sometimes significant, inhibitory effect on spore germination.

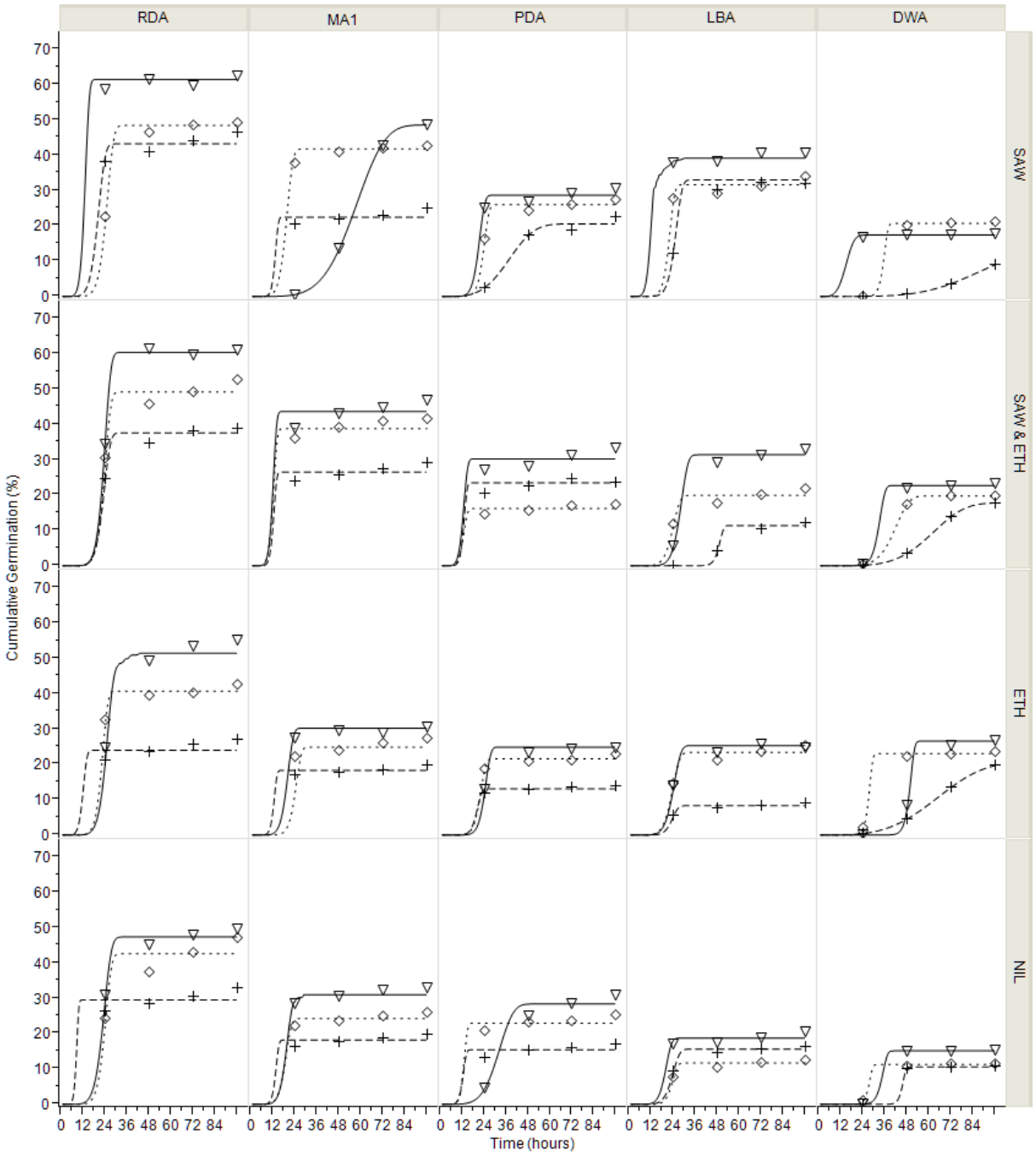

Figure 3.2. Cumulative percentage germination of *Ganoderma mastoporum* basidiospores, plated at 3 × 104 spores mL-1, over a 96 hour period, fitted by the Weibull-type model, Equation 1.1. Effects of five different nutrient media: Rice Dextrose Agar (RDA); 1% Malt Extract Agar (MA1); Potato Dextrose Agar (PDA); Lima Bean Agar (LBA); Distilled Water Agar (DWA) from left to right, in approximate order of decreasing germination. The effect of the additive: sawdust (SAW), 2% ethanol (ETH), both sawdust and ethanol (SAW & ETH) or none (NIL), is presented, from top to bottom, in combination with the medium in approximate order of decreasing germination success. Points represent observed data at three different incubation temperatures ◇ 22 ▽ 27 + 31, as the means of four replicates across two experimental runs. Error bars are not shown for visual clarity. Curves represent fitted models at three constant incubation temperatures signified by ···22 —27 - - -31.; see Table S3.3 for parameter estimates and goodness of fit.

Ganoderma philippii

The results for *G. philippii* were very different from those for the other species (Figure 3.3). Malt Extract Agar (MA1) with sawdust plus ethanol as additives resulted in a cumulative germination incidence having a maximum of 51% at 27°C, with no other treatment combination reaching 40% germination. As with *G. mastoporum*, germination was not observed at either 10°C or 35°C, but the shape of the germinating curve was more often like that of *G. australe* (i.e. gradually sigmoidal) rather than that of *G. mastoporum* (i.e. steeply sigmoidal). This difference can largely be attributed to the delay in germination of *G. australe* and *G. philippii*, both of which had zero or close to zero germination in the first 24 h, whereas *G. mastoporum*, in most cases, was already close to maximum germination percentage.

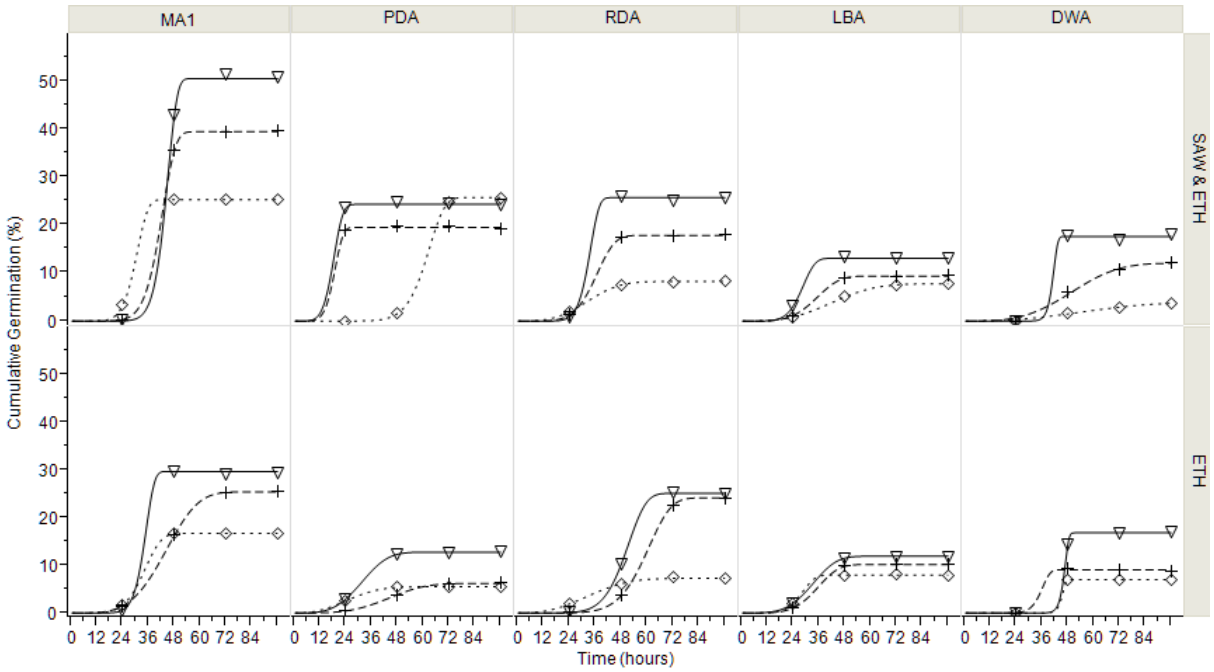

Figure 3.3. Cumulative percentage germination of *Ganoderma philippii* basidiospores, plated at 3 × 104 spores mL-1, over a 96 hour period, fitted by the Weibull-type model, Equation 1.1. Effects of five different nutrient media: 1% Malt Extract Agar (MA1); Potato Dextrose Agar (PDA); Rice Dextrose Agar (RDA); Lima Bean Agar (LBA); Distilled Water Agar (DWA) from left to right, in approximate order of decreasing germination. The effect of the additive: 2% ethanol (ETH) and both sawdust and ethanol (SAW & ETH) is presented, from top to bottom, in approximate order of decreasing germination success. Points represent observed data ◇ 22 ▽ 27 + 31, as the means of four replicates across two experimental runs. Error bars are not shown for visual clarity. Curves represent fitted models at three constant incubation temperatures signified by ····22 —27 - - -31; see Table S3.4 for parameter estimates and goodness of fit.

Weibull-type model assessment

The goodness-of-fit of the Weibull-type model (Equation 1.1) to the cumulative germination frequencies for the spore density 3×10^4 spores/mL is shown graphically (Figures 3.1, 3.2 and 3.3) and quantitatively (Tables S3.2, S3.3 and S3.4). Depending upon whether convergence was achieved or not, and whether that convergence was conditional, the results for parameters *M*, *b* and *c* fell into three broad groups. For the first group, the convergence criterion was met unconditionally. For these data sets, all parameter estimates are meaningful. In the second the predicted germination was the same for the last two, three or four times (i.e. 24, 48, 72 and 96 h) at which measurements were made. Here, only conditional convergence was obtained and only the least-squares estimate of the parameter *M* is meaningful. The third is where the convergence process did not get beyond step 0. In this case, an estimate of *M* was obtained as

the mean of the 16 observations at the times 24, 48, 72 and 96 h, and the standard error of the mean as the standard error of those 16 observations. For *G. australe*, 57 of 84 data sets converged unconditionally, compared with only seven of the 60 data sets for *G. mastoporum* and 19 of 30 data sets for *G. philippii*. Although mean values for the estimates of the parameters *b* and *c* for each species can be calculated from the values listed in Tables S3.2, S3.3 and S3.4 that converged unconditionally, caution is needed in drawing conclusions about the population values of these parameters because of the imbalance among the species that resulted from the large number of data sets that converged only conditionally or failed to converge.

3.3.2. Germination dynamics

For each *Ganoderma* species separately, only a small percentage of treatment combinations for basidiospore concentrations of 1×10^4 mL^{-1} and 8×10^4 mL^{-1} successfully germinated. Therefore, attention was focussed on the germination dynamics for basidiospore concentrations of 3×10^4 spores/mL only. Of the 100 treatment combinations for each *Ganoderma* species, successful germination at the temperature extremes of 10°C and 35°C was obtained only for a handful of runs for *G. australe* and never for *G. mastoporum* or *G. philippii*. We therefore now look only at the 60 treatment combinations for each species where the five nutrient media are combined with four media additives at the three most successful temperatures, viz. 22, 27 and 31°C. Table S3.5 reports the results of the ANOVA on parameter *M*, the parameter for which meaningful estimates were obtained for each treatment combination. For each species, there was a significant interaction ($P<0.01$) between medium and media additive, but the table reports the main effect means with a view towards seeing which treatment levels, on average, perform better or worse than others. We examine each species separately.

Ganoderma australe

The best overall nutrient medium is RDA, the best additive is sawdust, and the best temperature is 22°C, although the percentage germinated is not significantly different from that

at 27°C (Table 3.2). The best individual treatment combination is RDA plus sawdust at 22°C, with the same medium plus the same additive at 27°C coming second (Figure 3.1). The ANOVA of parameter *M* using 60 input values (5 media × 4 additives × 3 temperatures) resulted in one significant interaction, that being Medium*Additive (P=0.0063) with mean values for the treatment combinations given in Table S3.5. From that table, the highest overall mean percentage germinated is 57.9%, for the combination RDA with sawdust, averaged over temperature. Therefore, all indicators point to the best germination occurring on RDA with added sawdust, with temperatures of 22°C or 27°C being almost equally efficacious.

Ganoderma mastoporum

Results obtained were similar to those for *G. australe*, with RDA as the best individual nutrient medium, and sawdust the best additive when these factors are averaged over the levels of the other factors (Table 3.2). However, the optimum temperature is 27°C, with a clear lead over the second best temperature, 22°C. Ethanol provides neither benefit nor detriment for *G. mastoporum*, the overall germination mean being unchanged compared to the control (NIL), whereas for *G. australe* it appears to have a depressive effect (Table 3.2). This is particularly noticeable in Figure 3.1, where the combination of RDA and ethanol resulted in a much-reduced germination success compared with RDA coupled with the other additives, including the control. Another striking difference between germination success for *G. australe* and *G. mastoporum* is that the response curves of the latter approach the asymptote more quickly than the former, as expressed through the scale parameter *b*. The difference is best observed by comparing the curves in Figure 3.2, with their steeply rising slopes, against the curves in Figure 3.1, which rise more gradually towards the asymptote. The graphs for *G. mastoporum* (Figure 3.2) confirm that germination has almost reached its asymptote by 48 h, with a substantial proportion of the asymptote reached at 24 h, whereas for *G. australe*, germination has scarcely begun at 24 h (Figure 3.1). The ANOVA of parameter *M* using 60 input values (5 media × 4 additives × 3 temperatures) gave significant effects of Medium ($P<0.0001$), Additive

(*P*<0.0001), Temperature (*P*<0.0001), Medium*Additive (*P*=0.0012), and Medium*Temp (*P*=0.0142). The mean values for the treatment combinations are given in Table S3.5, which support the conclusion from Table 3.2 that the combination of the best individual treatments (RDA, SAW and 27°C) results in the highest germination rate.

Ganoderma philippii

Results in Table 3.2 for the separate factors reveal major differences between the cumulative germination successes from those of the other two *Ganoderma* species. Whereas RDA was best for both of the other species, MA1 was better than any other nutrient medium for *G. philippii*. Another major difference is that no germination was observed unless ethanol was present, either with or without sawdust. The ANOVA of parameter *M* using 60 input values (5 media × 4 additives × 3 temperatures) gave significant effects for Medium (*P*<0.0001), Additive (*P*<0.0011), Temperature (*P*<0.0002), and the Medium*Additive interaction (*P*=0.0027). Mean values for the treatment combinations are given in Table S3.5, which confirm that the best overall treatment combination (MA1, SAW&ETH, 27°C) is what one would obtain by combining the best individual treatments from Table 3.2.

Table 3.2. Main effect means for nutrient medium, medium additive, and incubation temperature on the maximum germination (%), as estimated by parameter *M* of the Weibull-type model, of *Ganoderma australe*, *G. mastoporum* and *G. philippii* basidiospores, plated at 3 × 104 spores/mL. The sample size n indicates the number of values in the mean for that treatment group. DNG denotes a result where no germination was recorded and hence the data could not be modelled; the asymptote *M* was recorded as zero in this case.

Treatment		***M* (percent)**		
		G. australe	***G. mastoporum***	***G. philippii***
Medium[a]	RDA	[d]38.1[a]	44.9[a]	9.0[b]
(*n* = 12)	MA1	29.5[b]	30.8[b]	15.6[a]
	LBA	26.2[bc]	22.4[c]	5.0[d]
	PDA	24.0[c]	22.6[c]	7.9[bc]
	DWA	16.3[d]	18.2[d]	5.6[cd]
Additive[b]	SAW	39.6[a]	33.1[a]	DNG[c]
(*n* = 15)	SAW & ETH	26.9[b]	30.0[b]	20.1[a]
	NIL	23.9[b]	22.9[c]	DNG[c]
	ETH	17.0[c]	25.1[c]	14.4[b]
Temperature[c]	22°C	30.0[a]	27.9[b]	5.8[c]
(*n* = 20)	27°C	27.7[a]	34.3[a]	11.4[a]
	31°C	22.8[b]	21.2[c]	8.7[b]

[a]Medium: MA1 = 1% Malt Extract Agar; PDA = Potato Dextrose Agar; RDA = Rice Dextrose Agar; LBA = Lima Bean Agar; DWA = Distilled Water Agar. [b]Additive: NIL = none; SAW = 10g dried sterile sawdust; ETH = 2% ethanol; SAW & ETH = 10g dried sterile sawdust + 2% ethanol. [c]Temperatures = 22, 27 and 31°C. [d]Means followed by the same lower-case letter in a column depicting a treatment for each *Ganoderma* species are not significantly different (Fisher's LSD, α=0.05)

3.3.3. Effects of simple vs. complex carbohydrates

Each of the three *Ganoderma* species was able to germinate on the entire range of nutrient media; however, germination was always higher on media amended with an additional carbohydrate source compared with the control (Figures 3.1, 3.2 and 3.3).

3.4. Discussion

The Weibull-type model provided a good fit to the germination data for all three *Ganoderma* species for all nutrient media, media additives and temperatures. Combined with their relatively

low values of the root mean square error, these results indicate that the Weibull-type model is suitable for modelling cumulative spore germination data in *Ganoderma* species, and support the Weibull-type model as a biologically relevant means of describing the course of spore germination under each set of conditions, also permitting accurate reconstructions of germination over time for comparison among species.

In each *Ganoderma* species, combinations involving spore densities of 1×10^4 spores/mL and 8×10^4 spores/mL had a lower cumulative germination percentage after 96 h than at the optimal spore concentration of 3×10^4 spores/mL. Spore concentration is a critical factor in the germination of many kinds of fungal spore (Feofilova *et al.* 2004), and low spore densities may be disadvantageous for the conditioning of the spores themselves. On the other hand, high spore densities can also hinder germination through production of self-inhibitors that affect germination of neighbouring spores (Fries 1984). This is in keeping with the theory that germinating spores produce stable metabolites which can retard or inhibit germination of spores of the same species, and yet they may also produce metabolites which stimulate germination (Garrill 1995). An equilibrium between stimulatory and inhibitory effects is suggested here, as an optimal concentration of metabolites for rapid spore germination is frequently found in mycological studies in which the germination time is plotted for a range of spore concentrations (Feofilova *et al.* 2012). *Ganoderma philippii* responded differently to spore concentration than did *G. australe* and *G. mastoporum.* In the latter two species, there was no delay of germination onset when basidiospores are plated at 1,000 spores/cm^2 compared with 380 spores/cm^2, as is the case with *G. philippii.* This indicates that subsequent basidiospore germination is inhibited by the germinating basidiospores rather than the ungerminated basidiospores as appears to be the case for *G. philippii.* At the lower spore concentration, germination onset is delayed in all three species, indicating that all three produce some unknown substance that promotes basidiospore germination.

Each of the three species achieved highest percentage germination on different media and it is possible that this reflects individual species preferences for different carbon sources; however, the media are all complex and it is uncertain which component of each medium was responsible for stimulated germination. Autoclaving agar produces an organic acid that can inhibit spore germination of some ectomycorrhizal basidiomycetes (Bjurman 1984; Bjurman and Fries 1984), so it is possible that the addition of other compounds to the agar provides a protective rather than stimulatory effect *per se*. The apparent difference in carbon preference shown by these species of *Ganoderma* is perhaps understandable when considering the substrates that form their best suited habitats. Both *G. australe* and *G. mastoporum* are generally observed to have low pathogenicity yet compete well, via strong saprotrophic ability in soil and organic debris, forming sufficient heterokaryotic inoculum to persist (Mohammed *et al.* 2014). In contrast, *G. philippii* appears to be aggressively pathogenic but a poor competitor in the saprophytic stage (Mohammed *et al.* 2014).

No germination was observed in *G. philippii* unless ethanol was present, either with or without sawdust. Although ethanol stimulates basidiospore germination of *G. lucidum* (Srivastava *et al.* 2010), it has not been reported as an essential compound. Cochrane et al. (1963) have suggested that it might serve in pure culture as a precursor of some more commonly required compound, e.g. an amino acid; amino acids are known to occur in the soil adjacent to plant roots (Schroth and Hildebrand 1964). Alternatively, the ethanol may act as a signal to break dormancy in a similar manner to insect ingestion (Lim 1977). It is unclear how significant such insect ingestion is in nature, or whether some compound in wood can perform this function, despite the fact that the addition of sawdust to the media never resulted in successful germination in the absence of ethanol in this study. The addition of sawdust to DWA enabled the germination of *G. lucidum* basidiospores (Srivastava *et al.* 2010) but not of the *G. philippii* basidiospores in the current study. It is possible that insect ingestion may modify the sporoderm sufficiently to allow germination (Lim 1977) and that the ethanol may perform

a similar function (Srivastava *et al.* 2010). This behaviour of *G. philippii* is strikingly different from *G. australe* and *G. mastoporum*, which germinated without the aid of ethanol, and for which the addition of sawdust was clearly significantly better than ethanol alone.

For each *Ganoderma* species, the three most successful temperatures were 22, 27 and 31°C. For *G. mastoporum* and *G. philippii*, the maximum percentage germination was obtained at 27°C, with significantly lower germination incidences at both higher and lower temperatures. For *G. australe*, the optimum temperature is less clear, as the percentage germination at 22°C and 27°C did not differ significantly (Table 3.2). As germination at the temperature extremes of 10°C and 35°C was obtained occasionally for *G. australe*, compared with never for *G. mastoporum* or *G. philippii*, this may reflect the broader geographical and climatic range of *G. australe*, which occurs in temperate through to tropical regions.

This study has successfully demonstrated a simple method for the collection and germination of *G. australe*, *G. mastoporum* and *G. philippii* basidiospores. The availability of a simple method for axenic spore germination is necessary to obtain monokaryons and study mating systems in fungi (Billiard *et al.* 2012). The ability to produce monokaryon isolates will also facilitate population genetic studies based on mating type genes (Kile 1983; Pilotti *et al.* 2003) and development of molecular markers to enable further investigations of the ecology and dispersal mechanisms of the three species in this study (e.g. Anderson *et al.* 2006; Franzén *et al.* 2007; Nakabonge *et al.* 2005). In particular, understanding the population genetics of *G. philippii* and knowledge of spore germination dynamics will contribute to a greater understanding of the modes of infection of this pathogen and its spread in *A. mangium* and *E. pellita* plantations.

Ganoderma phillipi is an intractable pathogen to control and to date the most promising means of management appears to lie in biological control by competitive wood degrading fungi as for *Heterobasidion* spp. (Mohammed *et al.* 2014). Therefore this germination study also

paves the way for studies into competition between *G. philippii* basidiospores and oidia of potential biological control agents on stumps of felled trees, such as have been conducted in other pathosystems (Oliva *et al.* 2015; Sun *et al.* 2009).

3.5. Supplementary Material

Table S3.1. Nutrient media and media additives. Media are arranged according to their constituent soluble carbohydrate concentrations (g L^{-1}) and complexity. Media codes and additive codes are defined. Treatment preparation instructions are given.

Nutrient medium: carbohydrate concentration and complexity	**Plate preparation (25 ml aliquots were poured into 50 mm Petri dishes)**	**Mono-saccharide (g L^{-1})**	**Poly-saccharide (g L^{-1})**	**Medium code**
No added carbohydrates (control):				
Distilled Water Agar	20g Difco agar powder in 1 L distilled water, sterilise	0	0	DWA
Simple carbohydrates:				
Low: 1% Malt Extract Agar	33.6 g Difco MEA powder in 1 L distilled water, sterilise	10.4	2.75	MA1
High: Potato Dextrose Agar	39 g Difco PDA powder, 5g dextrose in 1 L distilled water, sterilise	25	4.1	PDA
Complex carbohydrates:				
Low: Rice Dextrose Agar	20 g Difco agar powder, 2.75 g dextrose, 16.2 g rice flour in 1 L distilled water, sterilise	2.75	11.3	RDA
High: Lima Bean Agar	Simmer 160 g *Phaseolus lunatus* seed in 500 ml dH_2O for 30 min, strain and add 20 g Difco agar powder to the liquid. Blend solids to a fine pulp, mix with liquid and increase volume with distilled water to make 1 L, sterilise	4.45	25.1	LBA

Media additives were prepared as per standard media, except 20 ml L^{-1} distilled water with 20 ml L^{-1} 100 % EtOH (post-sterilization, immediately prior to pouring) was substituted. Sawdust preparation: 10 g of dried sterile sawdust (prepared from a mixture of ground-up eucalyptus and *Acacia dealbata* wood and roots, and ground through a 2 mm mesh); this was added to the Petri dish prior to pouring media. Sterile 1M HCl or 1M NaOH was used to adjust medium to a pH of 6.0 ± 0.5 prior to pouring. The media additives were applied in all combinations with each of the five nutrient media. Additive codes: None (NIL); 2% ethanol (ETH), 10 g sterile sawdust (SAW) (prepared as above), 10g dried sterile sawdust + 2% ethanol (SAW & ETH).

Table S3.2. Evaluation of the germination dynamics of *Ganoderma australe* (at 3 x 10^4 spores mL^{-1}) basidiospores depending on nutrient medium, medium additive and temperature using the estimated parameters of the Weibull-type model, Equation 3.1.

Medium[a]	Additive[b]	Temp. (°C)	Parameter estimates[c] and respective approximate standard error (SE) values[d]			RMSE[e]
			M **(percent)**	***b*** **(hours)**	***c*** **(no units)**	
MA1	NIL	10	9.607 (1.58)	79.50	3.497	0.564
MA1	NIL	22	37.064 (1.23)	47.211	6.454	3.491
MA1	NIL	27	25.288 (0.73)	43.070	10.826	2.073
MA1	NIL	31	23.696 (0.71)	40.877	6.138	2.000
MA1	NIL	35	13.697 (0.68)	60.693	2.797	0.830
MA1	SAW	10	5.785 (0.19)	34.612	3.266	0.544
MA1	SAW	22	52.633 (1.21)	48.414	11.222	3.431
MA1	SAW	27	43.070 (1.05)	48.119	10.450	2.972
MA1	SAW	31	27.660 (0.88)	48.515	4.670	2.426
MA1	SAW	35	23.478 (0.91)	47.674	2.335	1.671
MA1	ETH	10	4.908 (0.24)	48.675	2.341	0.421
MA1	ETH	22	17.634 (0.43)	41.623	11.579	1.208
MA1	ETH	27	25.051 (0.58)	41.839	11.826	1.646
MA1	ETH	31	16.169 (0.64)	59.305	3.755	1.202
MA1	ETH	35	5.113 (0.12)	—	—	0.427
MA1	SAW&ETH	10	11.633 (0.37)	35.016	4.485	1.043
MA1	SAW&ETH	22	36.530 (1.16)	42.518	11.619	3.269
MA1	SAW&ETH	27	22.765 (0.59)	—	—	2.061
MA1	SAW&ETH	31	27.013 (0.86)	38.940	5.724	2.419
MA1	SAW&ETH	35	21.530 (0.50)	—	—	1.724
PDA	NIL	22	21.350 (0.67)	47.989	11.063	1.890
PDA	NIL	27	20.599 (0.58)	40.443	11.964	1.653
PDA	NIL	31	17.685 (0.48)	—	—	1.349
PDA	SAW	22	38.091 (0.91)	50.605	7.378	2.581
PDA	SAW	27	45.056 (1.35)	—	—	3.825
PDA	SAW	31	26.823 (0.76)	—	—	2.136
PDA	ETH	22	19.085 (0.60)	48.854	10.627	1.689
PDA	ETH	27	18.161 (0.49)	42.309	9.002	1.392
PDA	ETH	31	17.064 (0.47)	41.536	3.637	1.314
PDA	ETH	35	9.361 (0.33)	44.151	2.934	0.820
PDA	SAW&ETH	22	21.584 (0.50)	—	—	1.747
PDA	SAW&ETH	27	23.801 (0.58)	—	—	2.010
PDA	SAW&ETH	31	18.833 (1.03)	—	—	3.561
PDA	SAW&ETH	35	14.030 (2.11)	—	—	2.600
RDA	NIL	10	13.010 (9.76)	—	—	3.348
RDA	NIL	22	25.589 (1.66)	42.361	8.577	4.704
RDA	NIL	27	37.609 (2.62)	45.926	7.388	7.412
RDA	NIL	31	24.297 (1.22)	—	—	3.443
RDA	NIL	35	19.66 (1.02)	—	—	2.876
RDA	SAW	10	19.642 (1.40)	50.463	2.229	2.101
RDA	SAW	22	62.699 (3.27)	—	—	11.314
RDA	SAW	27	61.168 (2.79)	—	—	9.674
RDA	SAW	31	49.852 (2.10)	—	—	7.286
RDA	SAW	35	39.181 (1.51)	—	—	5.237
RDA	ETH	10	17.220 (1.93)	—	—	5.468
RDA	ETH	22	25.828 (1.55)	44.298	7.141	4.377

Medium[a]	Additive[b]	Temp. (°C)	Parameter estimates[c] and respective approximate standard error (SE) values[d]			RMSE[e]
			M **(percent)**	***b*** **(hours)**	***c*** **(no units)**	
RDA	ETH	27	23.414 (0.65)	41.987	4.553	1.832
RDA	ETH	31	20.376 (1.13)	46.481	10.913	3.186
RDA	ETH	35	21.310 (4.33)	60.370	2.064	3.073
RDA	SAW&ETH	10	36.271 (3.70)	73.693	8.034	7.368
RDA	SAW&ETH	22	48.161 (1.95)	48.189	12.165	5.507
RDA	SAW&ETH	27	42.628 (1.49)	40.789	7.712	4.204
RDA	SAW&ETH	31	35.541 (1.57)	47.706	10.720	4.431
RDA	SAW&ETH	35	27.175 (1.75)	45.674	10.581	4.952
LBA	NIL	22	29.484 (1.07)	42.695	11.461	3.039
LBA	NIL	27	23.578 (1.38)	—	—	4.777
LBA	NIL	31	21.270 (1.40)	—	—	6.288
LBA	NIL	35	6.534 (0.29)	36.631	3.087	0.822
LBA	SAW	22	43.348 (2.30)	—	—	7.950
LBA	SAW	27	34.721 (1.98)	41.488	9.661	5.606
LBA	SAW	31	35.406 (3.18)	—	—	8.999
LBA	SAW	35	15.986 (1.25)	42.660	4.982	3.524
LBA	ETH	22	24.002 (1.26)	41.422	4.428	3.554
LBA	ETH	27	17.323 (0.49)	—	—	1.380
LBA	ETH	31	17.103 (1.49)	—	—	5.146
LBA	ETH	35	7.218 (0.60)	45.445	2.004	0.977
LBA	SAW&ETH	22	24.824 (1.24)	44.634	9.850	3.512
LBA	SAW&ETH	27	27.206 (1.50)	—	—	4.254
LBA	SAW&ETH	31	15.891 (1.27)	41.569	5.303	3.594
LBA	SAW&ETH	35	5.180 (0.16)	38.281	4.092	0.453
DWA	NIL	22	17.648 (0.39)	67.129	7.308	0.787
DWA	NIL	27	13.720 (0.29)	—	—	0.832
DWA	NIL	31	19.048 (6.17)	89.141	4.604	0.709
DWA	SAW	22	30.253 (1.04)	57.593	7.144	2.089
DWA	SAW	27	23.488 (0.60)	48.281	10.112	1.693
DWA	SAW	31	19.232 (0.88)	68.676	4.790	1.544
DWA	SAW	35	10.559 (0.49)	49.271	2.279	0.816
DWA	ETH	22	3.051 (0.11)	77.178	7.162	0.155
DWA	ETH	27	4.864 (0.29)	—	—	0.831
DWA	ETH	31	6.023 (0.33)	69.889	3.683	0.354
DWA	SAW&ETH	22	20.949 (0.58)	42.393	6.986	1.6263
DWA	SAW&ETH	27	20.744 (0.54)	43.944	12.626	1.5231
DWA	SAW&ETH	31	16.479 (1.72)	71.349	7.553	3.4253
DWA	SAW&ETH	35	12.025 (0.32)	53.962	8.447	0.9097

The raw data are the mean of four replicates of each treatment combination across two experimental runs. [a]MA1 = 1% Malt Extract Agar; PDA = Potato Dextrose Agar; RDA = Rice Dextrose Agar; LBA = Lima Bean Agar; DWA = Distilled Water Agar. [b]Additives: NIL = none; SAW = 10g dried sterile sawdust; ETH = 2% ethanol; SAW&ETH = sawdust + 2% ethanol. [c]*M* = Maximum percent spore germination; *b* = Time until 63.21% of *M*; *c* = Shape parameter indicating the spread of germination in time; entered in the table as a long dash (—) when unconditional convergence of the model was not obtained. [d]Approximate standard error (SE) of parameter estimates from the true mean: not calculable for estimates of parameters *b* and *c* due to SAS PROC NLIN procedure returning singular Hessians. [e]Square root of the respective model's Error Mean Square (RMSE). Missing treatment combinations are where no germination was recorded and hence the data could not be modelled.

Table S3.3. Evaluation of the germination dynamics of *Ganoderma mastoporum* (at 3 x 10^4 spores mL^{-1}) basidiospores depending on nutrient medium, medium additive and temperature using the estimated parameters of the Weibull-type model, Equation 3.1.

Medium[a]	Additive[b]	Temp. (°C)	Parameter estimates[c] and respective approximate standard error (SE) values[d]			RMSE[e]
			M (percent)	*b* (hours)	*c* (no units)	
MA1	NIL	22	24.300 (0.70)	—	—	2.416
MA1	NIL	27	31.069 (0.65)	—	—	2.615
MA1	NIL	31	18.214 (0.46)	—	—	1.839
MA1	SAW	22	41.808 (1.06)	—	—	3.656
MA1	SAW	27	48.649 (2.05)	60.556	4.781	4.082
MA1	SAW	31	22.504 (0.55)	—	—	2.220
MA1	ETH	22	24.994 (0.56)	—	—	2.231
MA1	ETH	27	30.275 (0.72)	—	—	2.507
MA1	ETH	31	18.329 (0.35)	—	—	1.401
MA1	SAW&ETH	22	39.512 (0.74)	—	—	2.955
MA1	SAW&ETH	27	43.720 (1.03)	—	—	4.100
MA1	SAW&ETH	31	26.586 (0.65)	—	—	2.583
PDA	NIL	22	23.316 (0.54)	—	—	2.3147
PDA	NIL	27	28.455 (0.99)	33.424	5.256	2.8105
PDA	NIL	31	15.447 (0.39)	—	—	1.5518
PDA	SAW	22	26.001 (0.69)	—	—	2.3741
PDA	SAW	27	28.708 (0.74)	—	—	2.5490
PDA	SAW	31	20.577 (0.61)	40.694	3.795	1.7072
PDA	ETH	22	20.957 (0.43)	—	—	1.3085
PDA	ETH	27	24.938 (0.66)	—	—	2.2939
PDA	ETH	31	13.130 (0.32)	—	—	1.1122
PDA	SAW&ETH	22	16.241 (0.44)	—	—	1.5247
PDA	SAW&ETH	27	30.314 (0.88)	—	—	3.0638
PDA	SAW&ETH	31	23.530 (0.60)	—	—	2.4165
RDA	NIL	22	42.644 (1.19)	—	—	4.115
RDA	NIL	27	47.418 (1.11)	—	—	3.861
RDA	NIL	31	29.562 (0.71)	—	—	2.842
RDA	SAW	22	48.464 (0.85)	—	—	2.936
RDA	SAW	27	61.500 (1.81)	—	—	6.271
RDA	SAW	31	43.265 (1.05)	—	—	3.650
RDA	ETH	22	40.791 (1.09)	22.764 (2.41)	9.120	3.764
RDA	ETH	27	51.533 (1.20)	—	—	4.786
RDA	ETH	31	24.390 (0.59)	—	—	2.356
RDA	SAW&ETH	22	49.312 (1.20)	—	—	4.152
RDA	SAW&ETH	27	60.494 (1.47)	—	—	5.084
RDA	SAW&ETH	31	37.648 (1.09)	—	—	3.789
LBA	NIL	22	11.703 (0.29)	—	—	1.021
LBA	NIL	27	18.717 (0.54)	—	—	1.870
LBA	NIL	31	15.594 (0.39)	—	—	1.346
LBA	SAW	22	30.647 (0.78)	—	—	3.120
LBA	SAW	27	39.200 (1.16)	—	—	4.008
LBA	SAW	31	33.028 (0.81)	—	—	2.821
LBA	ETH	22	23.415 (0.55)	—	—	1.890
LBA	ETH	27	25.359 (0.64)	—	—	2.221
LBA	ETH	31	8.302 (0.18)	—	—	0.630
LBA	SAW&ETH	22	19.933 (0.54)	—	—	1.887

Medium[a]	Additive[b]	Temp. (°C)	Parameter estimates[c] and respective approximate standard error (SE) values[d]			RMSE[e]
			M **(percent)**	***b*** **(hours)**	***c*** **(no units)**	
LBA	SAW&ETH	27	31.450 (0.68)	—	—	2.353
LBA	SAW&ETH	31	11.400 (0.30)	—	—	0.845
DWA	NIL	22	11.238 (0.23)	—	—	0.807
DWA	NIL	27	15.138 (0.37)	—	—	1.295
DWA	NIL	31	10.598 (0.26)	—	—	0.727
DWA	SAW	22	20.689 (0.44)	—	—	1.512
DWA	SAW	27	17.412 (0.44)	—	—	1.527
DWA	SAW	31	12.397 (5.50)	90.816	4.527	0.518
DWA	ETH	22	23.062 (0.45)	—	—	1.549
DWA	ETH	27	26.598 (0.82)	—	—	2.324
DWA	ETH	31	20.815 (1.06)	—	—	0.947
DWA	SAW&ETH	22	19.795 (0.41)	42.946	6.932	1.154
DWA	SAW&ETH	27	22.726 (0.48)	—	—	1.666
DWA	SAW&ETH	31	17.830 (0.38)	65.799	4.747	0.718

The raw data are the mean of four replicates of each treatment combination across two experimental runs.
[a]MA1 = 1% Malt Extract Agar; PDA = Potato Dextrose Agar; RDA = Rice Dextrose Agar; LBA = Lima Bean Agar; DWA = Distilled Water Agar. [b]Additives: NIL = none; SAW = 10g dried sterile sawdust; ETH = 2% ethanol; SAW&ETH = sawdust + 2% ethanol. [c]*M* = Maximum percent spore germination; *b* = Time until 63.21% of *M*; *c* = Shape parameter indicating the spread of germination in time; entered in the table as a long dash (—) when unconditional convergence of the model was not obtained. [d]Approximate standard error (SE) of parameter estimates from the true mean: not calculable for estimates of parameters *b* and *c* due to SAS PROC NLIN procedure returning singular Hessians. [e]Square root of the respective model's Error Mean Square (RMSE). Missing data represent treatments where no germination was recorded and hence the data could not be modelled. There was no successful germination recorded at either 10°C or 35°C.

Table S3.4. Evaluation of the germination dynamics of *Ganoderma philippii* (at 3 x 10^4 spores mL^{-1}) basidiospores depending on nutrient medium, medium additive and temperature using the estimated parameters of the Weibull-type model, Equation 3.1.

Medium[a]	Additive[b]	Temp. (°C)	Parameter estimates[c] and respective approximate standard error (SE) values[d]			RMSE[e]
			M **(percent)**	***b*** **(hours)**	***c*** **(no units)**	
MA1	ETH	22	16.716 (0.47)	35.773 (6.58)	5.633	1.339
MA1	ETH	27	29.720 (0.72)	—	—	2.492
MA1	ETH	31	25.435 (0.70)	47.738 (2.84)	4.082	1.890
MA1	SAW&ETH	22	25.363 (0.70)	—	—	2.419
MA1	SAW&ETH	27	50.661 (1.34)	45.399 (2.08)	11.416	3.779
MA1	SAW&ETH	31	39.561 (1.16)	43.331 (4.17)	8.160	3.267
PDA	ETH	22	5.515 (0.16)	29.016 (2.00)	2.528	0.442
PDA	ETH	27	12.768 (0.45)	34.569 (2.84)	3.679	1.264
PDA	ETH	31	6.188 (0.18)	48.422 (1.54)	3.745	0.463
PDA	SAW&ETH	22	25.790 (0.83)	63.622 (2.08)	9.840	1.651
PDA	SAW&ETH	27	24.377 (0.66)	19.319 (1.61)	5.610	2.291
PDA	SAW&ETH	31	19.568 (0.63)	19.693 (1.81)	6.240	2.172
RDA	ETH	22	7.300 (0.23)	38.182 (2.01)	2.638	0.613
RDA	ETH	27	25.123 (0.63)	52.526 (10.13)	7.298	1.777
RDA	ETH	31	24.158 (0.56)	62.298 (1.21)	6.976	1.116
RDA	SAW&ETH	22	8.263 (0.24)	36.473 (1.91)	3.191	0.690
RDA	SAW&ETH	27	25.784 (0.56)	—	—	1.953
RDA	SAW&ETH	31	17.896 (0.51)	38.384 (2.54)	5.596	1.437
LBA	ETH	22	7.964 (0.23)	—	—	0.663
LBA	ETH	27	11.918 (0.33)	35.792 (2.30)	4.152	0.932
LBA	ETH	31	10.163 (0.36)	37.566 (2.92)	4.879	1.030
LBA	SAW&ETH	22	7.775 (0.19)	47.498 (1.44)	3.082	0.452
LBA	SAW&ETH	27	13.118 (0.28)	—	—	0.976
LBA	SAW&ETH	31	9.410 (0.29)	37.473 (2.32)	4.506	0.831
DWA	ETH	22	7.026 (0.23)	—	—	0.646
DWA	ETH	27	16.830 (0.43)	46.886 (0.25)	28.213	1.216
DWA	ETH	31	9.052 (0.26)	—	—	0.911
DWA	SAW&ETH	22	3.992 (0.47)	64.983 (7.22)	2.503	0.347
DWA	SAW&ETH	27	17.658 (0.55)	—	—	1.551
DWA	SAW&ETH	31	12.103 (0.55)	55.527 (2.61)	3.327	1.042

The raw data are the mean of four replicates of each treatment combination across two experimental runs. [a]MA1 = 1% Malt Extract Agar; PDA = Potato Dextrose Agar; RDA = Rice Dextrose Agar; LBA = Lima Bean Agar; DWA = Distilled Water Agar. [b]Additives; NIL = none; SAW = 10g dried sterile sawdust; ETH = 2% ethanol; SAW&ETH = sawdust + 2% ethanol. [c]*M* = Maximum percent spore germination; *b* = Time until 63.21% of *M*; *c* = Shape parameter indicating the spread of germination in time; entered in the table as a long dash (—) when unconditional convergence of the model was not obtained. [d]Approximate standard error (SE) of parameter estimates from the true mean: not calculable for estimates of parameters *b* and *c* due to SAS PROC NLIN procedure returning singular Hessians. [e]Square root of the respective model's Error Mean Square (RMSE). Missing data represent treatments where no germination was recorded and hence no data could be modelled. There was no successful germination recorded at either 10°C or 35°C, or where ethanol was not present as an additive.

Table S3.5. Means (±standard error, SE) of parameter *M* (percent) of the Weibull-type model for significant (P<0.01) treatment interactions (between medium borne soluble carbohydrates[a], medium additives[b], and incubation temperature[c]) of *Ganoderma australe, G. mastoporum* and *G. philippii* basidiospores, plated at 3 x 10^4 spores mL^{-1}.

Interaction	*G. australe* Mean (SE)	*G. mastoporum* Mean (SE)	*G. philippii* Mean (SE)
Medium x Additive (n=3)			
MA1 x NIL	28.7 (4.22)	24.5 (3.71)	DNG
MA1 x SAW	41.1 (7.27)	37.7 (7.83)	DNG
MA1 x ETH	19.6 (2.75)	24.5 (3.46)	24.0 (3.83)
MA1 x SAW&ETH	28.8 (4.07)	36.6 (5.16)	38.5 (7.32)
RDA x NIL	29.2 (4.24)	39.9 (5.34)	DNG
RDA x SAW	57.9 (4.05)	51.5 (5.84)	DNG
RDA x ETH	23.2 (1.58)	38.9 (7.89)	18.9 (5.79)
RDA x SAW&ETH	42.1 (3.65)	49.2 (6.60)	17.3 (5.07)
PDA x NIL	19.9 (1.12)	22.4 (3.78)	DNG
PDA x SAW	36.7 (5.31)	25.1 (2.39)	DNG
PDA x ETH	18.1 (0.58)	19.7 (3.47)	8.2 (2.31)
PDA x SAW&ETH	21.4 (1.44)	23.4 (4.06)	23.2 (1.88)
LBA x NIL	24.8 (2.45)	15.3 (2.03)	DNG
LBA x SAW	37.8 (2.77)	34.5 (2.71)	DNG
LBA x ETH	19.5 (2.26)	19.0 (5.39)	10.0 (1.14)
LBA x SAW&ETH	22.6 (3.44)	20.9 (5.81)	10.1 (1.58)
DWA x NIL	16.8 (1.59)	12.3 (1.42)	DNG
DWA x SAW	24.3 (3.21)	16.8 (2.41)	DNG
DWA x ETH	4.6 (0.86)	23.5 (1.68)	11.0 (2.99)
DWA x SAW&ETH	19.4 (1.46)	20.1 (1.42)	11.3 (3.97)
Medium x Temperature (n=4)			
MA1 x 22°C	36.0 (7.16)	32.7 (4.65)	10.5 (6.32)
MA1 x 27°C	29.0 (4.71)	38.4 (4.59)	20.1 (12.36)
MA1 x 31°C	23.6 (2.64)	21.4 (1.99)	16.2 (9.81)
RDA x 22°C	40.6 (9.08)	45.3 (2.11)	3.9 (2.25)
RDA x 27°C	41.2 (7.80)	55.6 (3.64)	12.7 (7.35)
RDA x 31°C	32.5 (6.61)	33.7 (4.19)	10.5 (6.20)
PDA x 22°C	25.0 (4.39)	21.6 (2.07)	7.8 (6.13)
PDA x 27°C	26.9 (6.16)	28.1 (1.13)	9.3 (5.86)
PDA x 31°C	20.1 (2.27)	18.2 (2.37)	6.4 (4.61)
LBA x 22°C	30.4 (4.48)	21.4 (3.93)	3.9 (2.27)
LBA x 27°C	25.7 (3.63)	28.8 (4.47)	6.3 (3.62)
LBA x 31°C	22.4 (4.48)	17.1 (5.52)	4.9 (2.83)
DWA x 22°C	18.0 (5.65)	18.7 (2.58)	2.8 (1.71)
DWA x 27°C	15.7 (4.16)	20.5 (2.59)	8.6 (4.98)
DWA x 31°C	15.2 (3.12)	15.4 (2.37)	5.3 (3.12)
Additive x Temperature (n=5)			
NIL x 22°C	26.2 (3.36)	22.6 (5.71)	DNG
NIL x 27°C	24.2 (3.90)	28.2 (5.65)	DNG
NIL x 31°C	21.2 (1.28)	17.9 (3.17)	DNG
SAW x 22°C	45.4 (5.65)	33.5 (5.11)	DNG
SAW x 27°C	41.5 (6.21)	39.5 (7.85)	DNG
SAW x 31°C	31.8 (5.19)	26.4 (5.35)	DNG
ETH x 22°C	17.9 (4.01)	26.6 (3.59)	8.9 (1.99)
ETH x 27°C	17.8 (3.55)	31.7 (5.04)	19.3 (3.51)
ETH x 31°C	15.3 (2.44)	17.0 (2.84)	15.0 (4.06)
SAW&ETH x 22°C	30.4 (5.25)	29.0 (6.53)	14.2 (4.69)
SAW&ETH x 27°C	27.4 (3.94)	37.7 (6.61)	26.3 (6.50)

SAW&ETH x 31°C	22.8 (3.76)	23.4 (4.41)	19.7 (5.30)

The raw data are the mean of four replicates of each treatment combination across two experimental runs. Entries in the table for medium x additive are averages of these means taken over the three temperatures 22, 27 and 31°C; entries for medium x temperature are averages of these means taken over the four additives; entries for additive x temperature are averages of these means taken over the five media. [a]Media: MA1 = 1% Malt Extract Agar; PDA = Potato Dextrose Agar; RDA = Rice Dextrose Agar; LBA = Lima Bean Agar; DWA = Distilled Water Agar. [b]Additives: NIL = none; SAW = 10g dried sterile sawdust; ETH = 2% ethanol; SAW&ETH = sawdust + 2% ethanol. DNG denotes a treatment where no germination was recorded and hence the data could not be modelled. Its value is taken to be zero for the calculation of means.

CHAPTER 4. SEXUALITY AND MATING TYPES OF *GANODERMA PHILIPPII, GANODERMA MASTOPORUM* AND *GANODERMA AUSTRALE*, THREE BASIDIOMYCETE FUNGI WITH CONTRASTING ECOLOGICAL ROLES IN SOUTH-EAST ASIAN PULPWOOD PLANTATIONS

Abstract

Species of *Ganoderma*, particularly *G. philippii, G. australe* and *G. mastoporum*, are commonly found in Indonesian *Acacia mangium* plantations. *Ganoderma philippii* is a root rot pathogen while the other two species are secondary root invaders and wood rotters. Management of *G. philippii* can be supported by knowledge of its gene flow, genetic diversity and population dynamics. This investigation was undertaken to determine the sexuality and mating systems of *G. philippii* and co-occurring *Ganoderma* species, observing the somatic interactions between monokaryotic and dikaryotic mycelia and noting any incompatibility mechanisms. In all three species monokaryons were self-sterile. By examining the contact-zone hyphae, it was determined that in all three species, full sexually compatible matings occurred in 26–33% of the crossings. Two mating type loci were identified, as is the case for a wide range of Basidiomycetes. Dikaryons generated from monokaryotic isolates showed morphological changes as cultures aged. The results of this study indicate that outcrossing is favoured in all three species, *G. australe, G. philippii* and *G. mastoporum*, therefore promoting adaptation to new hosts and environments.

4.1. Introduction

4.1.1. *Ganoderma* spp. in *Acacia mangium* plantations

Ganoderma philippii has been identified as the dominant pathogen causing red root-rot disease in *Acacia mangium* (Coetzee *et al.* 2011; Mohammed *et al.* 2012; Yuskianti *et al.* 2014), a key industrial species for pulpwood production in Indonesia (Arisman and Hardyanto 2006).

Red root rot is considered an economically damaging disease (Eyles *et al.* 2008; Potter *et al.* 2006). *Ganoderma mastoporum* and *Ganoderma australe* have also been associated with *A. mangium* in Sumatra (Glen *et al.* 2009); the former has been isolated from roots (Yuskianti *et al.* 2014) but in the absence of pathogenicity tests, it is unclear whether this species can act as a primary pathogen or is merely a secondary coloniser of damaged roots. We reject the synonymy proposed by Wang et al (2014) for *G. mastoporum* and *G. boninense* as even their own data shows high DNA sequence variation between *G. boninense* and *G. mastoporum/G. cupreum* and the oil palm pathogen does not, to our knowledge, occur on hardwoods. It is, however, possible that *G. cupreum* is synonymous with *G. mastoporum* (Glen *et al.* 2009). *Ganoderma australe* represents a diverse species complex that is widespread across both the northern and southern hemispheres (Moncalvo and Buchanan 2008); it is known as a decay agent of dead wood but not a root pathogen.

4.1.2. Pathogen life cycles and disease management

Management and prevention of basidiomycete stem and root rots in forest systems is generally achieved by integrating breeding programs for the development of resistant host cultivars and high risk site avoidance with targeted applications of silvicultural, chemical and biological control treatments (Cleary *et al.* 2013; Eyles *et al.* 2008; Laflamme 2010; Möykkynen *et al.* 2000; Möykkynen and Pukkala 2011; Susanto *et al.* 2005; Susanto *et al.* 2009). Although growers of *A. mangium* can learn from accumulated knowledge and experience of basidiomycete root-rot diseases in other forest systems, direct transference of practices known to reduce inoculum potential in temperate (Woodward *et al.* 1998) and tropical (Eyles *et al.* 2008; Nandris *et al.* 1987a) tree crops have met with little success, e.g. planting techniques, root excision and isolation trenching, stump removal and thinning (Ariffin *et al.* 2000; Pratt 2001; Pratt 1998), fungicide drenching, stump urea (Pratt 2001; Pratt 1998; Redfern *et al.* 2001; Redfern *et al.* 2010) and *Trichoderma* antagonism (Sundram *et al.* 2008; Susanto

et al. 2005). The poor level of control in *A. mangium* plantations is almost certainly due to the non-specificity of the treatments tested and exacerbated by poor understanding of the pathosystems involved. Economic considerations also severely limit the feasibility of many control strategies (Pratt 1998). For example, the practice of individually removing diseased trees and the underlying soil in high-value crops such as oil palm and rubber may not effectively target the primary mode of spread in pathogens associated with *A. mangium*. Regardless, these intensive approaches to controlling root disease are not economically viable to low-value pulpwood forestry applications (Mohammed *et al.* 2014).

Disease management may be enhanced by a thorough understanding of the pathogen, and knowledge of its biology and aetiology (Chee 1990; Cooper *et al.* 2011; Sariah 2003). For example, elucidating the genetic structures of *G. boninense* populations in oil palm revealed high levels of genetic diversity within and between plantations, strongly implicating basidiospore dispersal in disease progression (Miller 1995; Pilotti 2005; Pilotti *et al.* 2018; Pilotti *et al.* 2003). Basidiospores are now considered to be the primary means of disease spread, by direct infection of cut fronds and indirect root infection via colonized debris. New infections from mycelial contacts do occur, but far less frequently than was previously thought (Rees *et al.* 2009; Rees *et al.* 2011; Sanderson 2005). Whilst disease control by the development of resistant material and methods to reduce inoculum at replanting continue to be pursued, management strategies now also involve routine removal of basidiocarps, particularly in first rotation plantings with low levels of infection and few basidiocarps (Hunt and Pilotti 2004).

4.1.3. Genetics of sexuality, and mating systems of *Ganoderma* spp.

A knowledge of the sexuality, gene flow, genetic diversity and population dynamics of *Ganoderma* is prerequisite to determining the primary mode of root rot disease infection and spread in *A. mangium* plantations, as has been achieved for *G. boninense* (Sanderson 2005; Sanderson and Pilotti 1997; Sanderson *et al.* 2000). Studies in *G. lucidum* (Triratana and

Chaiprasert 1991), *Ganoderma tsugae* Murr. (Adaskaveg and Gilbertson 1986), *Ganoderma collosum* Hseu, *Ganoderma microsporum* Hseu, *Ganoderma fornicatum* Fr. (Pat.) (Hseu and Wang 1996) and *G. boninense* (Pilotti *et al.* 2002; Pilotti *et al.* 2003) have revealed that these species possess a tetra-polar mating system with alleles for heterothallism at two loci. Although Rajchenberg (2011) reports this finding as a strong characteristic feature of basidiomycetes, it should not be assumed that all species of the same genus have the same genetics of sexuality. The sexuality of *G. philippii, G. mastoporum,* and *G. australe* are unknown. Lim (1977) conducted studies on basidiospore germination of *G. philippii* but could only germinate the basidiospores following passage through the digestive system of an insect and did not publish any mating studies. Until recently, germination of *G. philippii* has remained problematic (Page *et al.* 2017) and this may have prevented such studies in this species.

Genomic sequence analyses have elucidated the genetic architecture of basidiomycete mating systems (James *et al.* 2013) but determination of mating type using molecular markers is not as reliable as using pairing tests (Skrede *et al.* 2013). To date, this level of genomic information is not available for the three *Ganoderma* species in this study. In addition, population genetic studies, as planned for *G. philippii,* will be enhanced by demonstrating independent segregation of microsatellite markers in a set of monokaryotic offspring from a single, heterozygous parent. Knowledge of the genetics, key mode and loci of infection and the propensity to accumulate aggressiveness genes will provide aetiological and ecological insights to programs currently screening for host resistance and biological control agents.

This investigation was undertaken as the first step in a study of the population genetics of *G. philippii* on *A. mangium* in Sumatra, Indonesia. Its aim was to determine the sexuality and mating systems of *G. philippii, G. mastoporum,* and *G. australe.*

4.2. Materials and methods

4.2.1. Spore collection

Spores were collected opportunistically and non-destructively from basidiocarps at the following locations:

- Langgam, Lat. 0.13ºN, Long 101.6 ºE: two *G. philippii* basidiocarps (from separate stumps, approximately 1 km apart in a stand of *E. pellita*); BO22947, BO22948. Spore collections were made from a third sporocarp in this area in April 2014 but the sporocarp was not collected.
- Logas, Lat. 0.29ºS, Long 101.27 ºE: one *G. mastoporum* basidiocarp (growing in a mature-age stand of *A. mangium*); BO22949.
- Baserah, Lat. 0.21ºS, Long. 101.48ºE: two *G. australe* basidiocarps (from two mature-age *A. mangium* wildling trees, approximately 750 m apart in a mature-age stand); BO22945, BO22946.

Following overnight collection of basidiospores onto filter paper (Page *et al.* 2017) spores were air-dried for 15 minutes before storage at room temperature in sealed, opaque containers. The basidiocarps were removed from the tree, placed in paper bags, dried for two weeks and then vacuum sealed for storage.

Basidiospore suspensions were incubated in antibiotic solution for 4 hours (Page *et al.* 2017), except for the *G. philippii* basidiospores that were collected in April 2014, which were incubated in antibiotic solution for 24 h. Spore density was calculated using an Optik-Labor Neubauer Improved 0.0025 mm^2 haemocytometer and aliquots diluted to 3×10^4 spores ml^{-1} (Page *et al.* 2017) .

4.2.2. Spore germination and single spore isolation

Petri dishes were prepared using optimal media for germination in 50mm Petri dishes (Page *et al.* 2017). Spores of *G. philippii* were pipetted onto 1% malt agar plus 2% ethanol (20 g agar, 10 g malt extract in 980 mL dH20, 20 ml technical grade 98% ethanol added post-sterilisation

prior to pouring). *G. australe* and *G. mastoporum* were pipetted onto rice dextrose agar (20 g agar, 5 g dextrose, 106 g rice flour in 1 L dH_20). For each species, 250 µl spore suspension was spread over the agar surface. Plates were sealed with parafilm and incubated in the dark at 27°C (*G. philippii* and *G. mastoporum),* or 22°C (*G. australe)* (Page *et al.* 2017).

Each plate was assessed for germination every 24 h under 50× phase contrast magnification using a Zeiss Axioskop. Single and suitably spaced germinated spores were cut from the agar surface by using a sterile hypodermic needle and placed, one to a plate, onto 3% potato dextrose agar (47 g Difco PDA in 1 L dH_20,). Plates were sealed and re-incubated in the dark at the above temperatures. After 6-7 days, the cultures had reached a size of approximately 1.5-2.0 cm diameter. Confirmation of monokaryon isolation was achieved by removing a small portion of mycelium and inspecting for clamp connections under 100× phase contrast magnification: isolates lacking clamp connections were subcultured separately onto PDA, placed again in dark incubation and allowed to grow to 7 cm diameter.

4.2.3. Species confirmation

Ganoderma philippii and *G. mastoporum* basidiocarps and isolates were confirmed by species specific PCR (Yuskianti *et al.* 2014) and *G. australe* basidiocarps and isolates by PCR and rDNA ITS sequencing (Glen *et al.* 2014).

DNA was extracted from sporocarps and fresh mycelium from single spore isolates. Sporocarp material, approximately 20 mg dry weight, was ground by hand with a plastic pestle under liquid nitrogen. Mycelial samples were ground with a plastic pestle and a Kontes motorised pellet mixer in a 1.5-ml microcentrifuge tube. A total of 250 µl extraction buffer (Raeder and Broda 1985) was added and the tubes incubated at 65°C for one hour. Tubes were centrifuged at 14,000 rpm for 15 min and the supernatant removed. DNA was extracted and purified

according to Yuskianti et al. (2014). DNA was eluted in 20 µl of TE buffer and an aliquot diluted 1/10 in TE for PCR.

4.2.4. Pairing technique and scoring

To study sexual incompatibility, pairings were made as per Adaskaveg (1986) and Pilotti (2002) with three replicates per pairing. Mycelial plugs of 5 mm^2 were cut from the growing edge of each isolate and placed approximately 1 cm apart in a petri dish containing 18 ml of 3% PDA (47 g Difco), and incubated in the dark at 25°C. Plates were examined at 10 days, and then every 7 days, for five weeks. At each assessment, a 2 mm^2 sliver of mycelia was removed from the confrontation zone and examined under 100× phase contrast magnification for the presence of clamp connections. All pairings were scored for pigmentation and line formation after three weeks and again after eight weeks incubation.

Two major pairings were performed: i) single-spore isolates from a single basidiocarp (intra-basidiocarp pairings) were paired in every possible combination, i.e. ten homokaryons harvested from each of two *G. philippii*, one *G. mastoporum* and two *G. australe* basidiocarps; ii) single-spore isolates representatives of each mating type, as determined by (i) from each basidiocarp were paired with representatives of each mating type from the other basidiocarp of the same species (inter-basidiocarp, intra-species pairings). Successful anastomoses were characterized by complete fusion of hyphal walls, protoplasm continuity and occurrence of nuclei in the middle of hyphal bridges. Mating types were assigned to each monokaryon according to patterns of compatibility (Esser 1962; Esser 1971; Miller *et al.* 1999; Pilotti 2005; Pilotti *et al.* 2002; Raper 1953).

4.2.5. Fluorescence microscopy

Quantitative evaluation of the nuclear condition of the cells of isolate pairings was made using the fluorescent stains DAPI (4' ,6-diamidino-2-phenylindole, Sigma-F6057,

Fluoroshield™ with DAPI; Sigma-Aldrich) and calcofluor (Fluorescent Brightener No. 28, Sigma-Aldrich). The DAPI illuminated the nuclei while the calcofluor illuminated the cell walls and cross-walls, facilitating the counting of nuclei in each cell.

Mycelial blocks of 2 mm^2 were placed 3.5 cm apart (to allow room for changing objectives) in the centre of glass slides covered with a thin layer of malt extract agar. Slides were sealed in petri dishes and incubated in the dark at 25°C until hyphal contact and interaction between isolates were observed. Excess moisture was removed by incubating the slides for 20 min at 35°C, the slides were then immersed in an 0.5 µg/ml aqueous solution of DAPI for not less than 30 minutes. Each slide was gently rinsed in distilled H_2O, then counterstained for five seconds with a 0.25% aqueous solution of calcofluor, immediately before examination. The slide was again rinsed, then mounted in the DAPI solution and left for 15 min at room temperature before viewing (Prigione and Marchisio 2004). Fluorescence images were acquired using a Leica Leitz DM RBE fluorescence microscope (A4 filter cube for UV light excitation BP 340–380 400 LP 425) fitted with a Leica DC300F digital camera interfaced to the Leica AF software suite (Leica Microsystems GmbH, Zetzlar, Germany). Image acquisition was under auto-contrast with exposure times between 2-4 s.

4.3. Results

4.3.1. Germination and mycelial interactions

Spores of all *Ganoderma* species germinated after 24-48 hours of incubation on agar media, except for those from the third *G. philippii* sporocarp, which were plated at lower density and took up to two weeks to germinate. DNA testing confirmed all putative single-spore isolates as either *G. philippii, G. mastoporum* or *G. australe* and were consistent with the basidiocarp from which the spores were collected. Isolates were examined for the presence of clamp connections and ten isolates lacking clamp connections were selected from each parent

basidiocarp for mating studies. Each of these isolates had white mycelium and there was no or very little pigmentation in the agar medium after 2 weeks' growth.

4.3.2. Intra-basidiocarp pairings

Ten to thirteen single-spore isolates from each individual basidiocarp were paired in all combinations. Six sets of pairings were performed; three from *G. philippii* basidiocarps, one *G. mastoporum* and two *G. australe* (Tables 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6). All pairings were examined for pigmentation and mycelial characteristics three and eight weeks after inoculation. The pairings of single basidiospore isolates from the third *G. philippii* sporocarp were conducted at a later date and observations were made weekly for 5 weeks.

Table 4.1. Mating reactions between sibling homokaryotic isolates 1-10 from *Ganoderma australe* fruiting body 2 (GAFB2)

GAFB2	A1B1			A2B2				A1B2		A2B1
	6	**3**	**5**	**10**	**4**	**9**	**2**	**8**	**7**	**1**
6	-	-	-	+	+	+	+	-	-	-
3		-	-	+	+	+	+	-	-	-
5			-	+	+	+	+	-	-	-
10				-	-	-	-	-	-	-
4					-	-	-	-	-	-
9						-	-	-	-	-
2							-	-	-	-
8								-	-	+
7									-	+
1										-

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned to different A and B alleles. Incompatible pairs were assigned common A and/or B alleles.
+ compatible; - incompatible (common A or AB alleles). 14/45 = 0.31 (31% sibling compatibility)

Table 4.2. Mating reactions between sibling homokaryotic isolates 11-20 from *Ganoderma australe* fruiting body 1 (GAFB1)

GAFB1	A3B3			A4B4			A4B3		A3B4	
	11	**16**	**13**	**17**	**12**	**18**	**14**	**19**	**15**	**20**
11	-	-	-	+	+	+	-	-	-	-
16		-	-	+	+	+	-	-	-	-
13			-	+	+	+	-	-	-	-
17				-	-	-	-	-	-	-
12					-	-	-	-	-	-
18						-	-	-	-	-
14							-	-	+	+
19								-	+	+
15									-	-
20										-

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned to different A and B alleles. Incompatible pairs were assigned common A and/or B alleles.
+ compatible; - incompatible (common A and/or B alleles). 13/45 = 0.29 (29% sibling compatibility)

Table 4.3. Mating reactions between sibling homokaryotic isolates 1-10 from *Ganoderma mastoporum* fruiting body 1 (GMFB1)

GMFB1	A5B5			A6B6				A5B6		A6B5
	8	4	1	9	3	6	2	7	10	5
8	-	-	-	+	+	+	+	-	-	-
4		-	-	+	+	+	+	-	-	-
1			-	+	+	+	+	-	-	-
9				-	-	-	-	-	-	-
3					-	-	-	-	-	-
6						-	-	-	-	-
2							-	-	-	-
7								-	-	+
10									-	+
5										-

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned to different A and B alleles. Incompatible pairs were assigned common A and/or B alleles.
+ compatible; - incompatible (common A and/or B alleles). 14/45 = 0.31 (31% sibling compatibility)

Table 4.4. Mating reaction between sibling homokaryotic isolates 1-10 from *Ganoderma philippii* Fruiting Body 1 (GPFB1)

GPFB 1	A7B7					A8B8		A7B8	A8B7	
	1	3	5	6	8	9	10	7	2	4
1	-	-	-	-	-	+	+	-	--	-
3		-	-	-	-	+	+	-	-	-
5			-	-	-	+	+	-	-	-
6				-	-	+	+	-	-	-
8					-	+	+	-	-	-
9						-	-	-	-	-
10							-	-	-	-
7								-	+	+
2									-	-
4										-

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned to different A and B alleles. Incompatible pairs were assigned common A and/or B alleles.
+ compatible; - incompatible (common A and/or B alleles). 12/45 = 0.27 (27% sibling compatibility)

Table 4.5. Mating reaction between sibling homokaryotic isolates 11-20 from *Ganoderma philippii* fruiting body 2 (GPFB2)

GPFB2	A9B9			A10B10				A9B10		A10B9
	13	16	20	18	12	19	11	14	15	17
13	-	-	-	+	+	+	+	-	-	-
16		-	-	+	+	+	+	-	-	-
20			-	+	+	+	+	-	-	-
18				-	-	-	-	-	-	-
12					-	-	-	-	-	-
19						-	-	-	-	-
11							-	-	-	-
14								-	-	+
15									-	+
17										-

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned to different A and B alleles. Incompatible pairs were assigned common A and/or B alleles.
+ compatible; - incompatible (common A and/or B alleles). 15/45 = 0.33 (33% sibling compatibility)

Table 4.6. Mating reaction between sibling homokaryotic isolates from *Ganoderma philippii* fruiting body 3 (GPFB3)

GPFB3	A11B11				A11B12			A12B11				A12B12	
	9-1	9-10	9-24	9-22	9-2	9-28	9-34	9-5	9-16	9-14	9-19	9-32	9-8
9-1	-	-	-	-	-	-	-	-	-	-	-	+	+
9-10		-	-	-	-	-	-	-	-	-	-	+	+
9-24			-	-	-	-	-	-	-	-	-	+	+
9-22				-	-	-	-	-	-	-	-	+	+
9-2					-	-	-	+	+	+	+	-	-
9-28						-	-	+	+	+	+	-	-
9-34							-	+	+	+	+	-	-
9-5								-	-	-	-	-	-
9-16									-	-	-	-	-
9-14										-	-	-	-
9-19											-	-	-
9-32												-	-
9-8													-

Compatible pairs (forming clamp cells, nuclear dikaryotisation) were assigned to different A and B alleles. Incompatible pairs were assigned common A and/or B alleles.
+ compatible; - incompatible (common A and/or B alleles). 20/68 = 0.29 (29% sibling compatibility)

The homokaryotic self-crosses showed consistent behaviour in each of the three replicates. As expected, self-pairings inevitably lacked line formation or colour change, with mycelia from the two agar plugs mingling freely. A wide variety of interactions was observed in the other pairings. Mycelial interactions between sibling monokaryons could be subjectively grouped

using observations of isolate macromorphology and hyphal interactions. Many pairings were characterised by a demarcation zone between the two isolates, starting as a zone of sparse growth. As the culture aged, mycelium covered the plate, but a ‘seam’ was apparent between the two isolates (Figure 4.1).

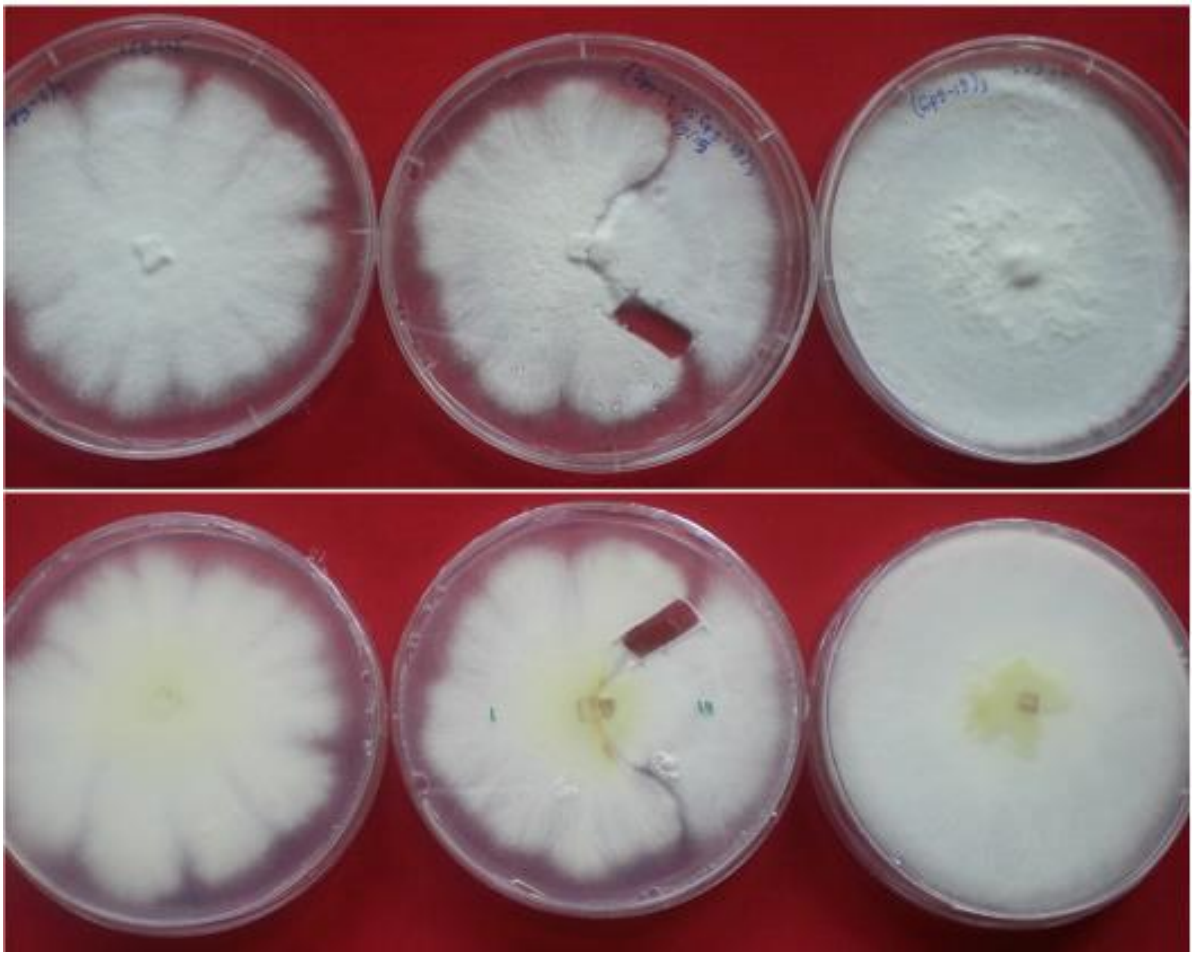

Figure 4.1. Pairing (centre) of incompatible *G. philippii* monokaryon isolates 9-1 (left) and 9-19 (right) as viewed from above (upper panel) and below (lower panel)

This demarcation was maintained after further subculture (Figure 4.2). In some pairings, colony morphology changed, from fluffy, off-white mycelium to a mottled, crustose, golden-brown appearance with strong yellow to golden-brown pigmentation in the agar. The first signs of pigmentation were observed at 2 weeks after inoculation, starting as lemon yellow to pale golden-brown and by five weeks the colour had deepened to a strong golden-brown (Figure 4.3).

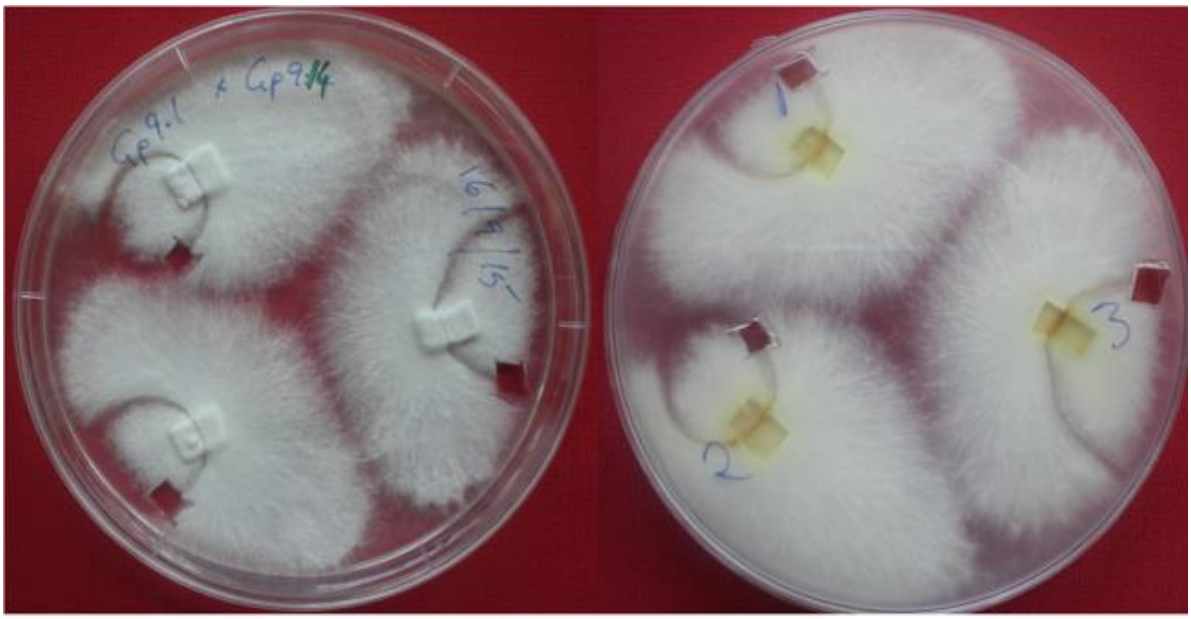

Figure 4.2. Three-week old subculture from the interaction zone of incompatible pairing 9-1 x 9-14, as seen from above (left) and below (right)

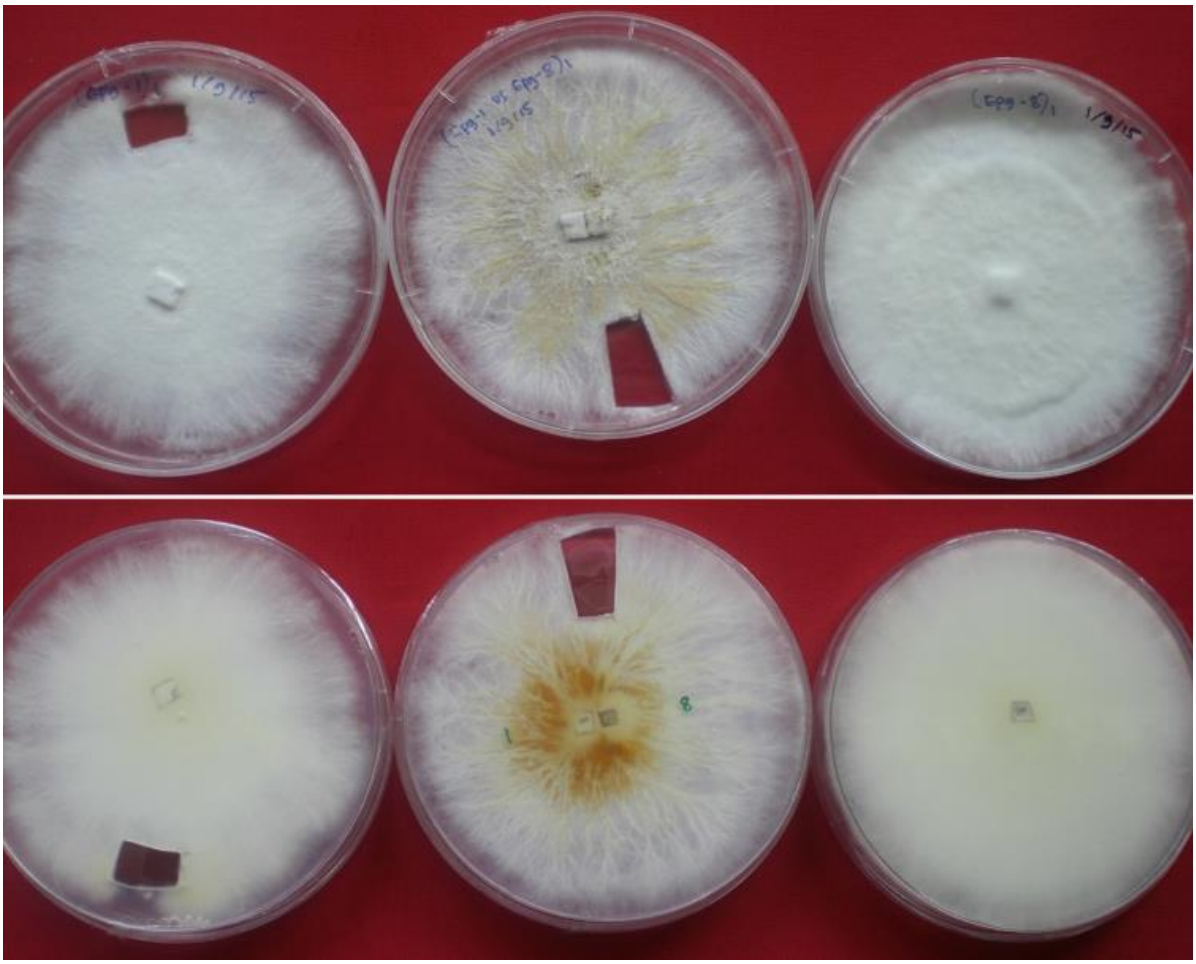

Figure 4.3. Pairing (centre) of compatible *G. philippii* monokaryon isolates 9-1 (left) and 9-8 (right) as viewed from above (upper panel) and below (lower panel)

Determination of compatibility based on the presence of clamp connections produced confusing results (data not shown), so the number of nuclei per cell was examined using fluorescent microscopy. This confirmed single nuclei per cell in single basidiospore isolates (Figure 4.4a). Examination of the mycelium from the interaction zone of paired isolates revealed that anastomosis occurred between lateral swellings of two neighbouring hyphae (Figure 4.4b; peg-to-peg fusion), two hyphal tips (tip-to-tip fusion), between a hyphal tip and a lateral hyphal wall (tip-to-side fusion), and between a hyphal tip and a lateral swelling of a hypha (tip-to-peg fusion). Upon fusion, clamp cells formed as projections of the hypha, enabling nuclear migration to occur by bridging the cell septa (Figure 4.4c and 4.4d). Clamp connections were absent in monokaryotic mycelia but were observed in many of the pairings.

Some pairings showed partial compatibility, with clamp cell formation; however, nuclear migration did not occur to complete dikaryotisation (Figure 4.4d). In fully compatible crosses, clamp cells formed, nuclei entered the mycelium of the opposite mating type (Figure 4.4e), the hyphal septa appeared to dissolve, and the nuclei migrated through the hyphae until they reached a tip cell.

Only the presence of clamp connections and two nuclei per hyphal cell after mating indicated full sexual compatibility. In all cases, monokaryons were self-sterile.

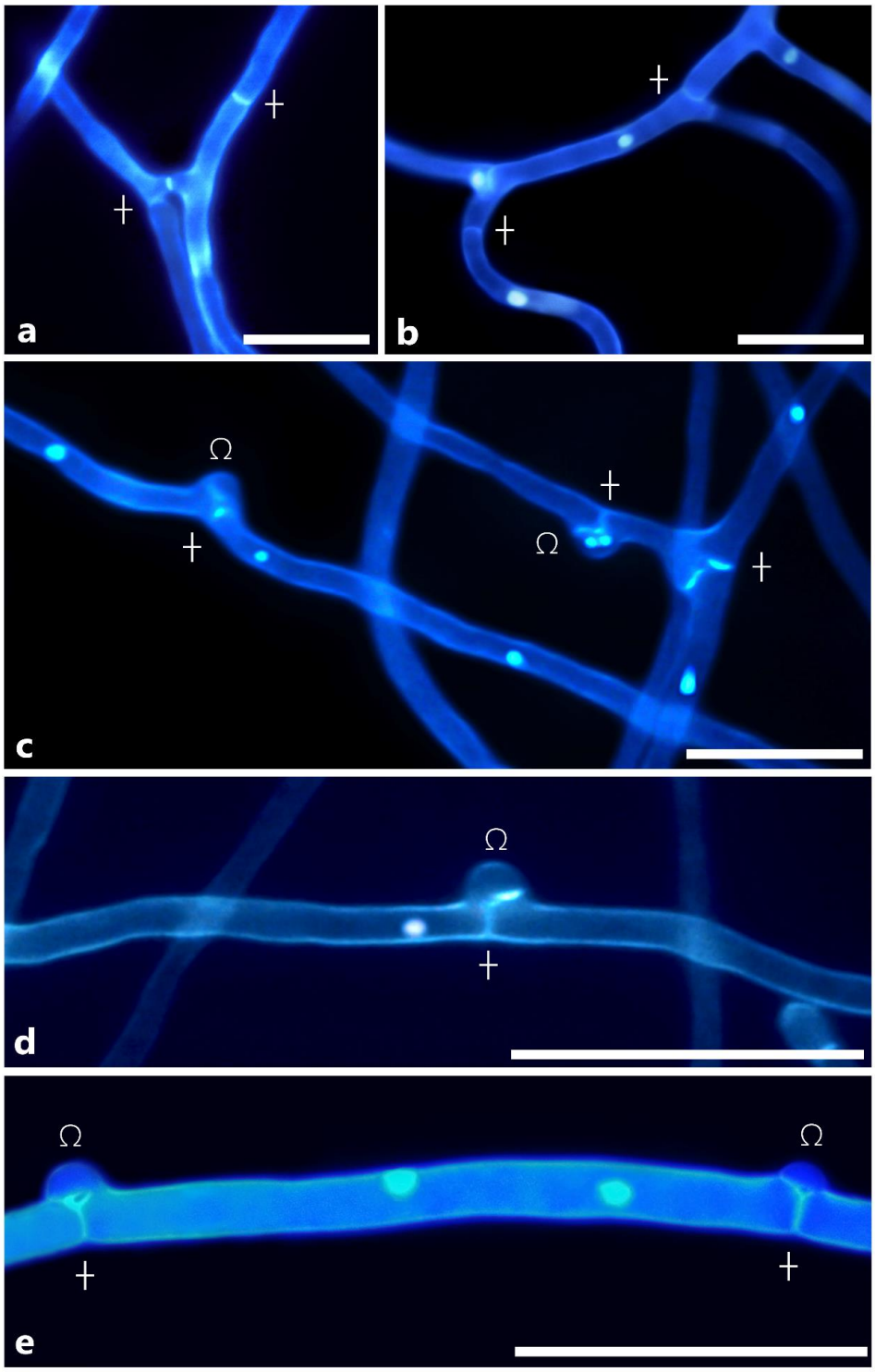

Figure 4.4. Photomicrographic overview of heterokaryon formation and nuclear status during axenic parings of G. philippii single spore isolates. a, sibling monokaryotic hyphae; showing a site of hyphal anastomosis resulting from the meeting of two lateral outgrowths (peg-to-peg fusion). b, monokaryotic hyphae, showing a single DAPI-stained nucleus per cell. c, dikaryotic hyphae; showing the site of hyphal anastomosis, clamp connections and two DAPI-stained nuclei per cell subsequent to migration. d, incompatible pairing; showing a clamp connection but no nuclear migration (one DAPI-stained nucleus per cell). e, dikaryon generated from the mating of fully compatible monokaryons, showing clamp connections and two DAPI-stained nuclei per cell. Clamp cells (Ω), septa (┼), Bar = 10µm.

The ratio of compatible to incompatible matings observed was ~1:4 in all three species. The percentage of compatibility varied from 26-33%, and single spore isolates could be categorised into groups representing mating types from each basidiocarp (Tables 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6). Sexually compatible monokaryotic parent isolates were arbitrarily assigned differing A and B mating type alleles. Partially compatible monokaryotic pairings were each assigned differing A alleles and identical B alleles. Incompatible monokaryotic parent isolates were assigned identical A and B mating type alleles (see Tables 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6).

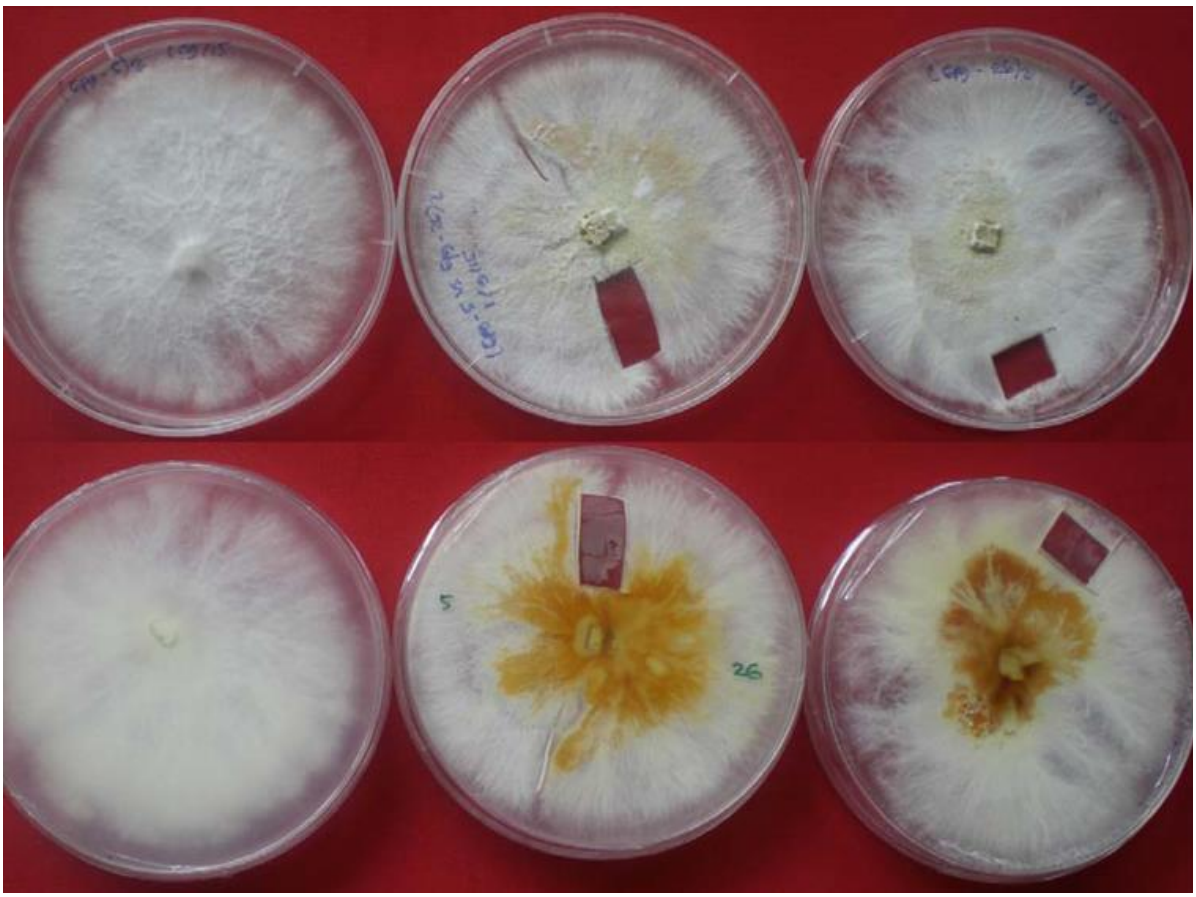

Figure 4.5. A compatible di-mon pairing between monokaryon 9-5 (A11B12, left) and dikaryon (right) reconstituted from monokaryons 9-14 (A11B12) and 9-2 (A12B11), as seen from above (upper panel) and below (lower panel)

Monokaryon isolates were also paired with reconstituted dikaryon isolates from the same parent. The monokaryon was dikaryotised when paired with a dikaryon containing a compatible nucleus. Gross morphological changes were similar to those observed in pairings between two monokaryons (Figures 4.5 and 4.6). The undikaryotised monokaryon grew more

slowly than the dikaryon though a zone of sparse growth often separated an incompatible monokaryon/dikaryon pairing (Figure 4.6).

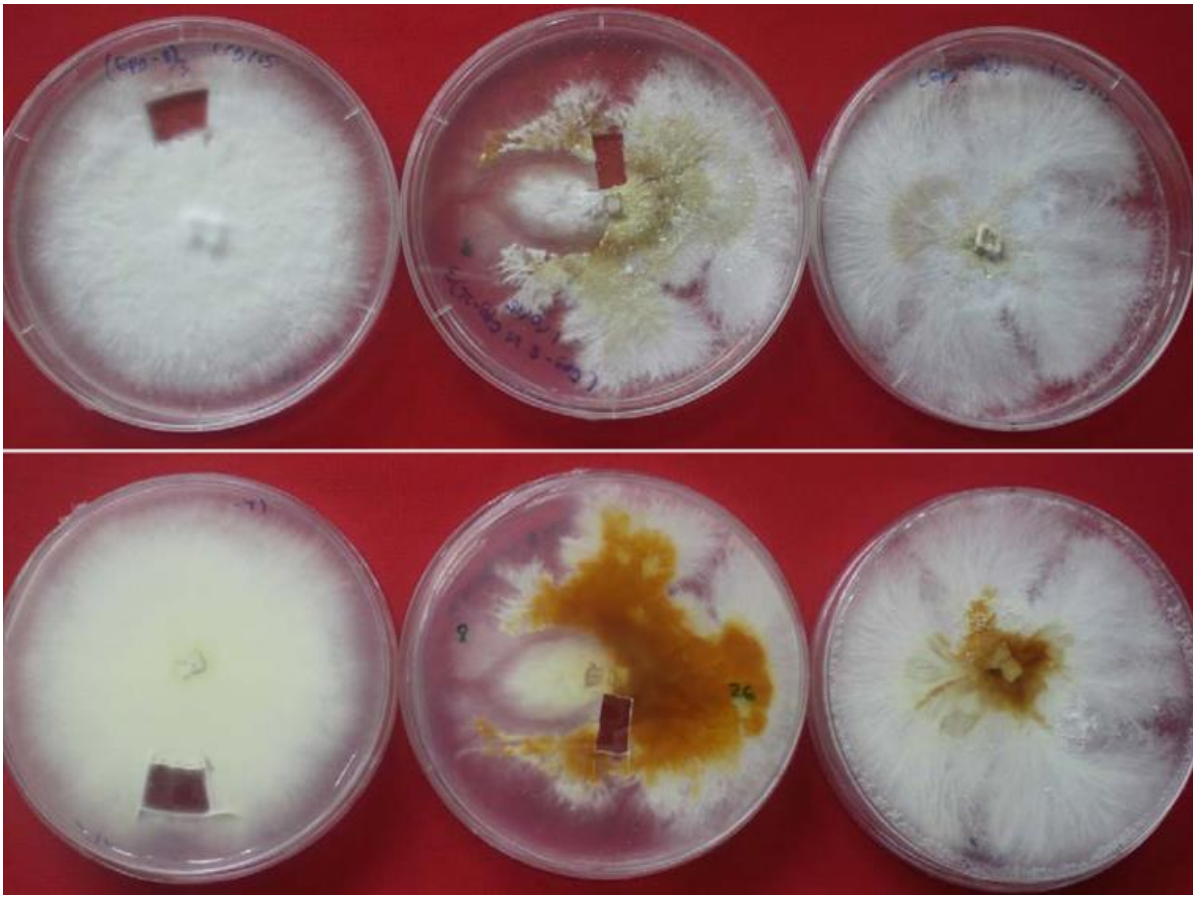

Figure 4.6. An incompatible di-mon pairing (centre) between monokaryon 9-8 (A12B12, left) and dikaryon (right) reconstituted from monokaryons 9-14 (A11B12) and 9-2 (A12B11), as seen from above (upper panel) and below (lower panel)

Changes in gross colony morphology (especially colour and texture) were common in compatible pairings but were not completely reliable as indicators of compatible pairings (Table 4.7). In compatible pairings, the upper surface changed from a fluffy texture with fine mycelial strands to a grainy crust with thick, ropey mycelial strands. The colour changed from white to yellow or golden-brown. There was often a strong yellow to deep golden-brown pigmentation on the underside of the agar. In incompatible pairings, the colour change was rare, took longer to appear and was usually paler or much smaller in extent.

Table 4.7. Comparison of macromorphology and scoring of clamp connections with mating reactions between sibling homokaryotic isolates from *Ganoderma philippii* fruiting body 3 (GPFB3)

GPFB3	A11B11				A11B12			A12B11				A12B12	
	9-1	9-10	9-24	9-22	9-2	9-28	9-34	9-5	9-16	9-14	9-19	9-32	9-8
9-1	-	-	-	-	-	c	c	-	-	-	-	CC	CC
9-10		-	-	-	-	-	-	-	-	-	-	CC	CC
9-24			-	-	-	c	c	-	-	-	-	CC	CC
9-22				-	-	-	c	-	-	-	-	CC	CC
9-2					-	c	-	CC	CC	CC	CC	-	-
9-28						-	-	CC	CC	CC	CC	-	c
9-34							-	CC	c	CC	CC	-	-
9-5								-	-	-	-	-	-
9-16									-	-	-	c	-
9-14										-	-	-	-
9-19											-	-	-
9-32												-	-
9-8													-

CC indicates abundant clamp connections (>5 per field of view), c indicates few clamp connections, - indicates no clamp connections seen. Grey shading indicates pairings that resulted in cultures with granular crusty cultures and yellow to golden-brown pigmentation in the agar.

4.3.1. Inter-basidiocarp pairings

For each species, monokaryons representing each mating type from each basidiocarp, were out-crossed in all combinations. In all cases full sexual compatibility, as indicated by the formation of clamps and nuclear migration in culture (data not shown), was recorded. Thus, it would seem as if multiple alleles exist at both mating type loci.

4.4. Discussion

Visual assessment of macroscopic growth form provided a preliminary indication of likely compatibility, as did the presence of abundant clamp connections, but both were less consistent and accurate than direct assessment of nuclear status by fluorescence microscopy. All three of the *Ganoderma* species in this study were heterothallic as demonstrated by changes in nuclear status and consistent with an estimated 90% of basidiomycete species (Kües *et al.* 2011). The pairing study confirmed this and the ~1:4 ratios of compatible to incompatible crosses observed between homokaryotic siblings are consistent with ratios found for other *Ganoderma* species (Adaskaveg and Gilbertson 1986; Hseu and Wang 1996; Pilotti *et al.* 2002; Triratana and

Chaiprasert 1991). Though sample sizes in this study were smaller than in many other studies, segregation of monokaryon sibling isolates into two groups with sexual incompatibility between the two groups indicates a tetrapolar mating system with mating type alleles at two loci (Esser 1962; Miller *et al.* 1999; Pilotti *et al.* 2002; Raper 1953). Genomic analyses have clarified the genetic basis of mating system determination in the Basidiomycota, and shown that the same genes are present in bipolar and tetrapolar species. These genes include the homeodomain (HD) encoding locus, also called the MAT-A locus, and the pheromone/receptor (P/R) locus, or MAT-B (James *et al.* 2008; James *et al.* 2013). A compatible mating requires heterozygosity at both of these loci and negative frequency-dependent selection results in high genetic variability of MAT alleles. The two loci are linked in bipolar species and unlinked in tetrapolar. Bipolarity is considered to be the ancestral state for fungi, whereas tetrapolarity is considered to be the ancestral state of Agaricomycetes, with bipolarity evolving on several occasions (James 2015; James *et al.* 2013). The assumption that this transition is irreversible (Raper 1953) has recently been questioned (James *et al.* 2013). The transition from tetrapolar to bipolar can occur by coalescence of the two MAT loci or by the evolution of a self-compatible pheromone/receptor pair, in either case all genes are still present allowing, theoretically, for reversion to tetrapolarity (James *et al.* 2013). A third cause of transition to bipolarity may be the formation of pseudogenes at incompatibility loci and reversion to terapolarity may be more difficult in this instance.

A tetrapolar mating system is the most complex of known fungal mating systems and is associated with both high outbreeding potential, and low inbreeding potential. Any one progeny can only mate with 25% of its siblings (Ni *et al.* 2011), affording an in-breeding restriction of 25% that favours out-crossing within a population (Ni *et al.* 2011). Outbreeding potential will depend on the number of alleles at each MAT locus in a population. In other tetrapolar basidiomycetes the number of alleles at the HD locus has been estimated from three

for *Crucibulum vulgare* to 288 for *Schizophyllum commune* (James 2015). At the P/R locus, the number ranges from two *(Tremella mesentericus)* to 354 *(Pleurotus populinus)* (James 2015). These can result in a high number of mating types, which can theoretically result in a very high outbreeding potential, but outbreeding is restricted by the locus with the fewer alleles. A total of 81 HD and 83 P/R alleles were detected from 52 dikaryons in a population of *G. boninense* from an oil palm plantation (Pilotti *et al.* 2003), in a study that clearly demonstrated the involvement of basidiospores in disease dissemination. A similar study looking at populations of *G. philippii* has potential to shed light on root-rot disease spread in *A. mangium* plantations in Indonesia, but fresh sporocarps of *G. philippii* are available for only a short period and most commonly produced on recently killed trees or those with advanced root disease. Sporocarps of *G. mastoporum* and *G. australe* are much more common than those of *G. philippii* in *Acacia* plantations in Indonesia (D. Puspitasari, pers. obs.). Multiple alleles appeared to be present in *G. philippii* and *G. australe* at both mating type loci, as shown by the mating compatibility of crosses between monokaryons from different basidiocarps. Given the relative ease of obtaining *G. philippii* isolates from infected roots (Francis *et al.* 2014; Yuskianti *et al.* 2014) compared to obtaining isolates from basidiospores, a population genetic study based on molecular markers is likely to be easier to implement than one based on pairing tests between monokaryons.

The three co-occurring *Ganoderma* species investigated in this study all produce a typical white rot of wood, though only *G. philippii* is an aggressive root pathogen. This species also varies in basidiospore production and germination. In addition to a lower abundance of sporocarps, and lower production of basidiospores per unit surface area (D. Page, pers. obs.) *G. philippii* is more particular in its germination requirements (Page *et al.* 2017).

Although spread and infection of *Ganoderma* in *A. mangium* plantations has historically been thought to occur clonally (Mohammed *et al.* 2012), there may be significantly more input

into these processes from basidiospores than has previously been considered. Population genetic studies are expected to provide further clarification, as has been the case for *Ganoderma* species in other crops such as oil palm (Pilotti *et al.* 2003).

CHAPTER 5. *ACACIA* PLANTATIONS IN INDONESIA FACILITATE CLONAL SPREAD OF THE ROOT PATHOGEN, *GANODERMA PHILIPPII*

Abstract

Ganoderma philippii is a root pathogen of many woody plants in tropical regions and is particularly aggressive to *Acacia mangium*, which is grown on a 6-year rotation for pulpwood in Indonesia. The disease becomes progressively worse over each rotation and control measures have met with limited success. We studied the population genetics of *G. philippii* to evaluate the role of sexual and asexual reproduction in its mode of spread. Populations were genetically distinct with high levels of inbreeding and clonal spread to adjacent trees increased after the first rotation. Despite the high levels of genetic diversity seen at all sampling scales, migration rates appear low. Measures to reduce the under-ground spread of the pathogen as well as methods to prevent the initiation of new infections from basidiospores will be needed to reduce the incidence of root rot in *A. mangium* plantations.

5.1. Introduction

Fungal species may reproduce predominantly by sexual or asexual methods, or alternate between these two modes of reproduction (Taylor *et al.* 2015). In pathogens of either animals or plants, sexual reproduction may facilitate, through genetic recombination, the development of strains or individuals that are highly aggressive against a particular host. Once established in a favourable environment, vegetative reproduction may expedite the production of large quantities of inoculum, enabling rapid colonization of new hosts or environments to which the pathogen has already adapted. The differing dispersal dynamics of sexual and asexual propagules will also result in different population genetic structures at different spatial scales (Barrès *et al.* 2012; Rieux *et al.* 2011). Mode of reproduction may vary depending on ecological

context and opportunity (Taylor *et al.* 2015). Dispersal processes are not always easy to infer within species, or between populations of the same species. Sexual reproduction may be cryptic (Saleh *et al.* 2012), or not very effective in producing new genotypes if inbreeding or selfing is common, and migration levels and distances are restricted (Dutech *et al.* 2008).

Molecular markers can assist in determining the level of genetic differentiation among subpopulations of forest pathogens. Population genetic analyses estimate the levels of sexual reproduction events within and between populations (Halkett *et al.* 2005). Analyses of genetic diversity at different spatial scales may reveal the geographic constraints on reproductive processes (Barrès *et al.* 2012; Dutech *et al.* 2008). Methods using spatial genetic analysis to estimate the genetic relatedness between individuals can be used to infer the dispersal of sexual and asexual propagules (Dutech *et al.* 2008; Rieux *et al.* 2011; Travadon *et al.* 2012). These population genetic studies can thus provide information about a pathogen's mode of spread and hence facilitate disease management.

Ganoderma philippii (Bres. & Henn. ex Sacc.) is a root pathogen of many tropical crops (Eyles *et al.* 2008) that is particularly damaging to *Acacia mangium* Willd., causing high levels of mortality particularly in second and subsequent rotations and a marked decrease in yield (Coetzee *et al.* 2011; Yuskianti *et al.* 2014). *Acacia mangium* is favoured for pulpwood production in Indonesia due to its high yield and low inputs (Arisman and Hardyanto 2006), though diseases including root rot and Ceratocystis wilt and canker have reduced its profitability (Mohammed *et al.* 2014). The integration of breeding programs for development of host resistance with avoidance of high risk sites and targeted application of silvicultural, chemical and biological control treatments have all been used to effect control of basidiomycete root rots in forest systems (Eyles *et al.* 2008; Laflamme 2010; Mohammed *et al.* 2014). Knowledge derived from experience of basidiomycete root-rot diseases in other forest systems may assist in the development of control measures against *G. philippii*, however little success

has been achieved to date using e.g. planting techniques, root excision and isolation trenching, stump removal and thinning, fungicide drenching, or stump treatment with urea (Woodward *et al.* 1998). Lack of specificity of the treatments and poor understanding of the pathosystem involved has contributed to inadequate outcomes. In addition, many of these strategies are not economically feasible (Pratt 1998). For example, the removal of diseased trees and the underlying soil may be appropriate in high-value crops such as oil palm and rubber but not in short rotation, low-value pulpwood forestry applications (see Mohammed *et al.* 2014). A thorough understanding of pathogen biology and particularly its dispersal and infection processes may assist in developing effective control measures (Cooper *et al.* 2011). *Ganoderma boninense* provides a useful example. This is a related pathogen that causes basal stem rot in oil palms, and population genetic studies have indicated a significant role for basidiospores in disease dispersal (Mercière *et al.* 2017; Mercière *et al.* 2015; Pilotti 2005; Pilotti *et al.* 2003). Studies have confirmed the ability of basidiospores to directly infect cut fronds and indirectly to infect roots via colonized debris (Rees *et al.* 2011). New infections from subterranean mycelial spread do occur, but far less frequently than was previously thought (Rees *et al.* 2011). Removal of basidiocarps has now been adopted as a routine practice, while efforts to develop resistant material and methods to reduce inoculum at replanting continue to be pursued (Hunt and Pilotti 2004). While these fundamental aspects of disease have been intensively studied in temperate species such as *Armillaria* (Heinzelmann *et al.* 2019) and *Heterobasidion* (Woodward *et al.* 1998), and tropical root rots in rubber (Suwandi *et al.* 2004) and oil palm (Cooper *et al.* 2011; Rees *et al.* 2011), relevant information is lacking for *G. philippii.*

Although spread and infection of *Ganoderma* in *Acacia* plantations has been thought to occur clonally (Mohammed *et al.* 2014), the involvement of basidiospores in dispersal and infection remains unknown (Page *et al.* 2018). Understanding dispersal mechanisms by

studying the population structure of *G. philippii* will inform management actions to minimise the impact of root rot. Currently, little is known about the dispersal of *G. philippii* in Acacia plantations. Outstanding questions include: Is genetic structure apparent within a landscape? Is clonal spread from tree to tree significant, as may be suggested by the low incidence of *G. philippii* basidiocarps (the sexual reproductive stage) in *Acacia* plantations in comparison with those of other *Ganoderma* species (Mohammed *et al.* 2014). Are disease centres dominated by a single genotype or by a suite of variable genotypes?

To begin to answer these questions, and to determine the extent of migration between populations, we conducted a population genetics study based on microsatellite markers. From these data, we were able to determine the spatial distribution of unique genotypes; calculate the amount of genetic variation within and between populations and sub-populations; and test for clonality and evidence of sexual recombination.

Our objectives were to assess the degree of genotypic diversity, clonality, and genetic differentiation in *G. philippii* populations within and among six plantation stands of *Acacia* sp. We specifically address the following questions regarding populations of *G. philippii* found in tropical *Acacia* plantations:

1) Do populations from different plantation compartments show genetic diversity and population structure or are they dominated by a single dominant or founding genotype?

2) Are infestations in subsequent rotations more genetically diverse than those in previous rotations, as might be expected if basidiospores play a major role in disease spread?

We evaluated two hypotheses.

1. Populations of *G. philippii* become increasingly and progressively more genetically diverse over successive rotations, indicating a role of basidiospores in dispersal.

2. Populations of *G. philippii* sampled in differing plantation stands are well differentiated, demonstrating a clear correlation between genetic and geographic distances.

5.2. Methods:

5.2.1. Locations

From 2006-2012, we sampled a total of 266 *Ganoderma philippii* isolates from five plantations in Sumatra, and one in Borneo, Indonesia (Figure 5.1), all planted to *Acacia mangium* and/or *A. auriculiformis*. Sites for monitoring plots were selected based on the rotation number and age of the trees (Table 5.1). Plots of 100 trees (10 rows x 10 trees) were centred on a diseased tree. Tree spacing was in most cases 3 m between rows and 2 m between trees within a row.

Figure 5.1. The plantations used in this experiment were at five locations in three provinces of Indonesia: Riau (Baserah, Langgam; Logas South); South Sumatra (Deras); and East Kalimantan (Sebulu).

Table 5.1. Site name, location, rotation and number of *Ganoderma philippii* isolates analysed.

Site name and number of plots	Co-ordinates	Rotation	Number of isolates	Plots sampled every 6 months between
Deras (3)	Lat. 3.31ºS, Long. 103.58ºE	1	38	2006-2008
Logas South 1 (3)	Lat. 0.29ºS, Long. 101.27ºE	1	52	2007-2009
Langgam (3)	Lat. 0.13ºN, Long. 101.6ºE	2	48	2006-2009
Logas South 2 (3)	Lat. 0.29ºS, Long. 101.27ºE	2	27	2011-2012
Baserah (3)	Lat. 0.39ºS, Long. 101.72ºE	3	51	2011-2012
Sebulu (4)	Lat. 0.01ºS, Long. 117.10ºE	3	50	2007-2009

5.2.2. Sampling

The main roots and associated secondary roots were exposed to a depth of approximately 10 cm and to a distance of 50 cm from the centre of the tree. A small nick was placed in the roots, just enough to peel off a very small section of the bark. If the root was healthy, it was fresh "greenish"-white; if infected, it was streaky, drying and pale brown. Where the red, rhizomorph-like skin characteristic of root rot caused by *G. philippii* was discovered, secateurs were used to excise a small sample from a secondary root which was placed in a sealable plastic bag, labelled and stored in a coolbox for transport to the laboratory and subsequent isolation attempt (Francis *et al.* 2014).

5.2.3. Isolations, culturing, and DNA extractions

Isolations were made according to Agustini *et al.* (2014a), on agar plates containing MAT (Malt Extract Agar with addition of (per litre) 50mg penicillin, 25mg polymyxin, 50mg streptomycin and 230 mg thiabendazole dissolved in 1 ml lactic acid) for most sites. For isolations at Logas South 2 and Baserah, a medium selective for *Ganoderma* was used, consisting of: Bacto-peptone, 5.0g; agar, 20g; $MgSO_4$, 0.25g; K_2HPO_4, 0.5 g; 95% ethanol, 20ml; Streptomycin Sulphate, 300 mg; Chloramphenicol, 100 mg; PCNB, 136 mg; Benlate-T, 150mg; tannic acid, 1.2 g; distilled water, 980 ml (as described in Ariffin and Idris 1990). Isolates were confirmed as *Ganoderma philippii* by species-specific PCR (Yuskianti *et al.* 2014) on DNA extracted from fresh mycelium (Agustini *et al.* 2014a).

5.2.4. Microsatellite Development

DNA was extracted from a Malaysian isolate of *Ganoderma philippii* using a Qiagen DNAeasy Plant minikit and sent to the Australian Genomic Research Facility for sequencing on a Roche 454 Sequencer following the standard GS-FLX Titanium library preparation (Gardner *et al.* 2011). The program MSATCOMMANDER version 0.8.1 (Faircloth 2008) was

used to search the resulting raw FASTA sequences for pure microsatellite motifs between 2 and 6 bp and with five or more repeats. Primers were designed with annealing temperatures close 60 °C and screened against two isolates to identify polymorphic loci.

5.2.5. Genotyping and data validation

Forward primers were synthesized with an additional 5' sequence (Blacket *et al.* 2012), i.e. TCAGGACCAGGCTACCGTG, to allow for amplification with a third, labelled, primer of that sequence. The third primer was labelled with a fluorescent phosphoramidite dye (Beckman Coulter). Microsatellite loci were amplified using Type-IT microsatellite master mix (Qiagen), forward, reverse and labelled primers at 0.10, 0.25 and 0.15 mM, respectively, with the addition of 0.1mg/ml BSA (Fisher Biotec). DNA was not quantified before PCR, though amplifiability was confirmed by amplification of the rDNA ITS region following Yuskianti *et al.* (2014). Microsatellite PCR products were electrophoresed on a Beckman Coulter CEQ 8000, following the manufacturer's recommended protocol and analysed using CEQ software (Beckman Coulter) with fragment sizes estimated by the inclusion of 0.5 µl DNA Size Standard Kit – 400 (Beckman Coulter) in each lane.

5.2.6. Marker amplification and null alleles

A total of 266 *Ganoderma philippii* isolates were obtained and subsequently genotyped with 11 microsatellite markers determined as polymorphic (Table 5.2). Null alleles were identified using indirect methods that account for excess homozygotes at null allele loci compared to other loci. As recommended by Dąbrowski *et al.* (2015), we combined the results of two programs, ML-NullFreq (Kalinowski *et al.* 2006) and Micro-Checker (Van Oosterhout *et al.* 2004). These two programs are complementary in the sense that Micro-Checker has a high detection rate associated with a high false-positive rate, while ML-NullFreq is less powerful but detects fewer false positives.

5.2.7. Uninformative loci / fixed alleles / missing data

The functions informloci and missingno from the R package *poppr* were used to check the data for quality issues including: identification of spurious allele calls, calculation of the percent missing data at the locus and genotype level, removing uninformative loci that contained one allele representing over 97% allele frequency and/or fixed alleles. Of the 85 alleles scored, 69 were retained for analysis.

Missing data might represent any source of error that could cause a PCR product to not amplify, which may or may not be biologically relevant. Basic per locus summary statistics of individual loci were checked for potential abnormalities in the data. Missing or null alleles were calculated per locus and assessed for indication of technical error during amplification. Descriptive statistics were produced, and analyses were conducted before and after selectively removing loci and/or samples with missing data to assess if statistical power and inference changed substantially.

To understand the nature of missing data in our data set before treatment, we took the following approaches:

- replacing missing data with zero, signifying a new (novel) allele
- replacing missing data with the mean allele frequencies in the entire data set
- removing missing data at the individual level, those with genotypes missing ≥5% data. Percent missing data for genotypes was considered the percent missing loci over number of total loci
- removing loci with ≥5% missing data (typically used for data sets that have systematic problems with certain loci that contain null alleles or simply failed to amplify)
- removing loci with ≥5% missing data AND subsequently all genotypes with ≥5% missing data

Of the 266 isolates genotyped, 216 had complete data at all nine loci, the remaining 50 had missing values at one locus. Thus all isolates were successfully genotyped at a minimum of 8 loci. This equated to a total average percentage of missing data of 1.04%. Plots at the Langgam site had the highest average missing data (1 - 5.1% of isolates per plot). Locus Phil30 stood out as the locus with the most missing data (5 - 46% of isolates per plot; 7.9% of all isolates) indicating a null allele(s) or technical error during amplification at this locus (Figure S5.1).

Table 5.2. Primer sequences for microsatellite loci. Note that forward primers had an additional 'tail' on the 5" end, consisting of the sequence TCAGGACCAGGCTACCGTG, to allow amplification with a third, fluorescently labelled, primer.

Locus	Forward primer	T_a (°C)[1]	Reverse primer	T_a (°C)[1]	Motif	No. repeats	Locus length	Allele size range
***phil*02**	GAAGACGAAGTGCGTCTTGG	54/60/54	GTCAGGGTCTGATTCAATTGC	52/60/51	ggga	6	98	101-121
***phil*09**	CTGCGGGCGAGGAGTTC	54/60/54	CCCTCTTGCCTAAGGATAGC	54/60/53	cttc	6	119	127-143
***phil*13**	GTTCGCTCTTCGATGAGACG	54/60/55	ACGCACGACGCACGAGC	54/60/58	cg	7	123	131-147
***phil*30**	GTACCGTGAGATACGAAGAGC	54/61/53	GAGGCACTTGCACAGGCAG	55/62/56	gtg	5	144	155-179
***phil*34**	GGATACCGTATACGGTATAGC	52/60/50	TGTACCTAATCGTCAGCTACG	52/60/53	cg	5	150	156-180
***phil*35**	TTCTGGTCGAATTACGACATCC	53/60/53	GCTCGGCAGATGGGAACC	55/61/54	gca	7	157	167-191
***phil*36**	ATTCACGCTTTGGAGAATGTGG	53/60/54	CTGGCGGACGTACCATGTC	55/62/54	cg	7	158	171-177
***phil*37**	GATCGTATCCCTCTAACCTTCG	55/62/54	TCCACTGCCACTTTGGAGGC	56/62/56	cgc	5	158	176-182
***phil*40**	GCCTTCTCGTATGTAGCTGC	54/60/54	AAGCCATCTCCTACGTCGTC	54/60/54	cg	5	163	171-293
***phil*51**	CCGTTCCATCGGACTCCG	55/61/54	TGTTGGAGGATCAATACGAGTG	53/60/53	gc	5	185	198-212
***phil*52**	TCTGCTGTAAATTCTCTGCCAG	53/60/55	GCAGCAATTGCTATCGGAGAC	54/61/56	ga	12	207	205-221
***phil*54**	CCTTCGCAGAGCCAGGC	54/60/55	CCCAAGACGTTTCAGTGCATC	54/61/54	ggga	6	98	101-121

[1]Basic/salt-adjusted/nearest neighbour, as per the Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html)

We determined the best approach to treat missing data was to assign the missing values to a novel allele size. Results were insensitive to this choice, showing; no reduction to statistical power of downstream analyses, sample size, 0% missing data, and no substantial changes to diversity indices (*H*, *G*, λ, or E_5). The novel allele method is commonly implemented in population genetics, as it carries a conservative assumption, and is the most parsimonious approach to treating missing states (Kamvar *et al.* 2014a).

5.2.8. Data analysis

Isolates were grouped into different multilocus genotypes (MLGs) using the R package *poppr* 2.2.0 (Kamvar *et al.* 2014a), which uses naïve string comparison to identify unique combination of alleles from the consensus of the nine simple-sequence repeat loci. A total of 205 unique MLGs were identified from the 266 isolates. For identification purposes, unique MLGs were assigned an arbitrary number from 1 to the total number of observed MLGs.

Naïve MLG definition simply takes strings of alleles and compares them for equality. The method is quick and easily interpretable, but means that things like genotyping error, hyper-variable loci, and missing data all contribute to a unique genotype that might not be truly unique (Kamvar *et al.* 2015). We defined MLGs for all sampled data. We implemented the method in the R package *poppr* (Kamvar *et al.* 2014a) for collapsing unique MLGs by genetic distance, allowing for incorporating genotypes that have missing data or genotyping error into their parent clusters, known as multilocus lineages (MLLs). We chose Bruvo's genetic distance (as it considers the real value of the alleles see Bruvo *et al.* 2004) and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA, or average neighbour) clustering algorithm. This algorithm clusters by creating a representative sample per cluster and joining clusters if these representative samples are closer than a given threshold, or put another way - merges clusters based on the average distance between every pair of points between clusters (Kamvar *et al.*

2015). The threshold is representative of the minimum genetic distance at which two individuals are from different clonal lineages and was defined using the *poppr* function cutoff_predictor. This function analyses the distribution of all possible thresholds to identify gaps in the distance distribution that represent clonal groups.

Genetic diversity

Population structure was studied by analysing allele frequencies, gene diversity, genetic distance, clonality, and genetic differentiation. Two types of data sets were generated for population genetic analyses: one that contained all MLLs from each population and the other that was a clone-corrected subset where every MLL was represented only once. Genetic diversity and population differentiation statistics were based on allele frequencies estimated from clone-corrected data, whereas genotypic diversity was estimated from complete data.

Population genetic analysis was conducted using the statistical language R (R Core Team 2018) using various packages as well as R functions written specifically for this project. Graphs and figures were created using the R packages *ggplot2*, *ape*, *igraph*, *ggmap,* and *poppr* (Csardi and Nepusz 2006; Kahle 2013; Kamvar *et al.* 2014a; Paradis *et al.* 2004; Wickham 2009).

Allelic diversity

The number, expected and observed heterozygosity, and evenness, of the alleles (within and across each locus) was analysed across and within sites and plots using the function locus_table from the R package *poppr* (Kamvar *et al.* 2014a) by calculating Nei's expected heterozygosity (Hexp Nei 1978), the observed heterozygosity (H_{obs}; number of heterozygotes / N) and allelic evenness (E5a, the ratio of the number of abundant alleles to the number of rarer alleles Grünwald *et al.* 2003).

Multilocus genotypic diversity

was analysed within and across sites, plots and rotations with the Shannon-Wiener index (*H*) and the Stoddard and Taylor's index (*G*), (Shannon 1948; Stoddart and Taylor 1988). Both *G* and *H* measure genotypic diversity, combining richness and evenness. If all genotypes are equally abundant, then the value of *G* will be the number of MLLs and the value of *H* will be the natural log of the number of MLLs. Both *G* and *H* are used because they weigh more or less abundant MLLs more heavily, respectively (Grünwald *et al.* 2003). Additionally, we calculated the Simpson index (λ), which is simply one minus the sum of squared genotype frequencies (Simpson 1949), equivalent to the expected heterozygosity. This measure provides an estimation of the probability that two randomly selected genotypes are different and scales from 0 (no genotypes are different) to 1 (all genotypes are different \Simpson, 1949 #4478}. Genotypic evenness was calculated as E_5b, which is an estimator of evenness that utilizes both *H* and *G* that gives a ratio of the number of abundant genotypes to rare genotypes (Grünwald *et al.* 2003). These were calculated with the R packages *poppr* and *vegan* (Kamvar *et al.* 2014a; Oksanen *et al.* 2013). Confidence intervals were calculated using the R package *aboot* with 9,999 bootstrap resamplings (Canty and Ripley 2015). Richness, or the expected number of MLLs (*eMLL*), was calculated using rarefaction from the R packages *poppr* and *vegan* (Kamvar *et al.* 2014a; Oksanen *et al.* 2013). The clonal fraction in each population was calculated as 1 – ((number of different genotypes) / (total number of isolates)). Because the analysis of genotypic diversity, richness, and evenness is agnostic to specific alleles within MLLs, assessment of genetic relatedness between MLLs was performed using the function bruvo.dist using *poppr*, which calculates Bruvo's genetic distance utilizing a stepwise mutation model for microsatellite loci (Bruvo *et al.* 2004; Kamvar *et al.* 2015; Kamvar *et al.* 2014b). Thus, this distance gives a more fine-scale picture of relationships between individuals than band-sharing models. These relationships were visualized with minimum spanning networks generated using the R packages *igraph* and *poppr* (Kamvar *et al.* 2015; Kamvar *et al.* 2014b).

Clonality

To assess the relative contributions of clonal reproduction and recombination, we used two approaches applied to both non-clone-corrected and clone-corrected data (Maynard Smith *et al.* 1993; Weir 1984). Both these methods assume that all alleles observed have the same evolutionary histories under clonality, but that alleles from different regions have different histories under recombination. First, associations among loci were tested by the index of multilocus linkage disequilibrium ($\bar{r}d$) as implemented in the R package *poppr* (Kamvar *et al.* 2015). Multilocus tests were not performed on sample sizes less than ten due to lack of statistical power (Hedrick 2011). The index has an expected value of zero if there is no association of alleles at unlinked loci, as expected in a randomly mating population. The significance of $\bar{r}d$ was tested by comparing the observed variance with the distribution of the expected variance under the null hypothesis of random mating, as determined from 1,000 randomizations of the genotype data. Second, the parsimony tree-length permutation test (PTLPT) derived from phylogenetic trees was used to determine significance by comparing the length of the tree of the observed data to the distribution of tree lengths of 1,000 permuted data sets (as described for $\bar{r}$d Burt *et al.* 1996). Phylogenetic trees were built from the MLLs using parsimony in PAUP version 4.0 (Stoddart and Taylor 1988) using an input file generated from GenAlEx 6.5 (Peakall 2012). For clonal populations, the observed tree length should be significantly shorter than the distribution of tree lengths under the null hypothesis of random mating.

Population structure

The genetic structure of the clone-corrected dataset was investigated using several complementary approaches.

Principle Component Analysis

We first performed a Principle Component Analysis (PCA) using the *ade4* R package (Dray and Dufour 2007). PCA allows a first visualization of the data, giving an idea of the overall sample structure.

Discriminant Analysis of Principal Components

Genetic differentiation was also investigated through the Discriminant Analysis of Principal Components (DACP Jombart *et al.* 2010) as implemented in ADEGENET version 2.1.1 (package used in R software, version 3.5.1 R Core Team 2018). DAPC does not rely on explicit population genetics models (such as minimising deviation from Hardy-Weinberg equilibrium) and is useful when the structure is subtle (Jombart *et al.* 2010). The *dapc* function is based on 2 steps: data are firstly transformed using a PCA, then a discriminant analysis (DA) is performed. As recommended in Jombart et al. (2010), for the *dapc* we retained a number of principal components that corresponded to more than ~90% of cumulated variance and all the linear discriminants.

Two methods for DAPC are available, depending on whether the number of origin samples (*K*) is known or not (with and without informative priors), and both were used in this study. In the first case (no information on *K*), before performing *dapc*, the function *find.clusters* was implemented to identify the optimal number of clusters. This function runs a *K*-means algorithm after transforming the original genotypic data into uncorrelated principle components through a Principle Components Analysis (PCA, a step done in order to reduce the number of variables and to speed up the clustering algorithm, Jombart and Collins 2017). *K*-means testing was run sequentially with increasing values of *K* from $K = 1$ to 40, with 1000 runs at each value of *K*, and different clustering solutions were compared using Bayesian Information Criterion (BIC). The optimal clustering solution is the one that corresponds to the

lowest BIC, indicated by a minimum in the curve of BIC values as a function of *K* (Jombart *et al.* 2010).

We also performed DAPC using the collection localities as prior groupings in an effort to explore geographic structuring at the plot and site level by maximising the multivariate distances between the respective sampling localities.

Mantel test for isolation by distance

A classical Mantel test was used to test for isolation by distance. We obtained a physical distance matrix using the *adegenet* R package to compute pairwise euclidean distances from the geographic data, and a genetic distance matrix using the function *bruvo.dist* using poppr, which calculates Bruvo's genetic distance utilizing a stepwise mutation model for microsatellite loci (Bruvo *et al.* 2004; Kamvar *et al.* 2014b). Thus, this distance gives a more fine-scale picture of relationships between individuals than band-sharing models. Correlation between physical and genetic distances was tested by performing Mantel tests across all hierarchical levels in the data set utilizing the function mantel.randtest in the R package *ade4* between Bruvo's distance as described above and Euclidean distances between geographic coordinates (Dray and Dufour 2007). *P* values were calculated using 99,999 bootstrap replicates.

Analysis of Molecular Variance

Population genetic structure was analysed further by conducting an analysis of molecular variance (AMOVA Excoffier and Quattro 1992). This approach is derived from the analysis of variance framework based on Wright's fixation indices as defined by Cockerham (1973). A hierarchical analysis of variance was conducted to partition variance into covariance components due to intra-individual, inter-subpopulation, and inter-population differences. Covariance components are used to calculate Φ fixation indices (Weir 1984). Significance of

fixation indices was tested using 2,000 nonparametric permutations. The analysis was performed twice: once with isolates grouped by plot and once with isolates grouped by site.

5.3. Results

A total of 266 *Ganoderma philippii* isolates (90 from first, 75 from second, and 101 from third rotation populations) were obtained from symptomatic acacia roots. All 11 microsatellite loci were amplified, but locus *phil*37 was eliminated from analysis because it had >10% null alleles that remained unresolved after rescreening. Another dinucleotide repeat locus (*phil*54) showed single-nucleotide polymorphisms, which did not allow accurate scoring of alleles, hence it was also eliminated. Thus, among the 11 markers applied in this study, 9 were suitable for analysis. These markers produced fragments of sizes consistent with expectations based on known repeat lengths, with less than 1.7% of null alleles at each locus (average 0.43%). All isolates were successfully genotyped at 7-9 polymorphic loci. From these, 205 unique MLGs were identified by naïve *s*tring comparison. The genotype accumulation curve indicated that we have enough statistical power within our data to detect 90% of multilocus genotypes (MLGs) with nine SSR markers. However, a typical plateau in the number of MLGs was not observed (Figure S5.2). The 205 unique MLGs were collapsed into MLLs by UPGMA clustering utilizing Bruvo's genetic distance (2004), set at a genetic distance threshold of 0.056. (Figure S5.3), which is equivalent of filtering genotypes that differ only by an average of a single mutational step (in a diploid organism with nine loci (0.25/9)*2, see Bruvo *et al.* 2004). The 205 MLGs previously defined by naïve string comparison were thus collapsed into 168 MLLs, with each assigned an arbitrary number from 1 to 168.

5.3.1. Population genetic analysis

Within locus allelic diversity

Allelic diversity across all loci did not significantly depart from Hardy Weinberg Equilibrium (H_{exp} = 0.46, H_{obs} = 0.43, see Table 5.3). Allelic diversity within loci revealed that *phil*34 had the highest number of alleles (n = 13). All other loci had eight or fewer alleles with a moderate to high amount of diversity, save for *phil*36, which had 4 alleles and very low diversity (H_{exp} = 0.14) (Table 5.3). Three loci (*phil*34, *phil*40, *phil*51) show significant differences between H_{exp} and H_{obs}, indicating they are not at Hardy Weinberg Equilibrium (HWE). This may be because the samples represent six distinct populations and most had private alleles. Alleles were relatively evenly spread, with those at *phil*09 close to equal in abundance (E_5a = 0.87), the overall mean relatively even at E_5a = 0.66, and locus *phil*36 slightly more dominated by a single allele at E_5a = 0.47 (Table 5.3).

Table 5.3. Allelic diversity metrics for each locus of clone-corrected *Ganoderma philippii* data across six *Acacia* plantation stands.

Locus	Alleles[1]	H_{exp}[2]	H_{obs}[3]	E_5a[4]
*phil*02	5	0.45	0.55	0.69
*phil*09	5	0.61	0.7	0.87
*phil*13	8	0.52	0.5	0.66
*phil*30	7	0.53	0.61	0.77
*phil*34	13	0.58	0.31	0.59
*phil*35	6	0.39	0.38	0.52
*phil*36	4	0.14	0.13	0.47
*phil*40	7	0.46	0.34	0.75
*phil*51	7	0.44	0.3	0.63
Mean	7.22	0.46	0.43	0.66

[1]Alleles = Number of observed alleles, [2]H_{exp} = Nei's expected heterozygosity (Nei 1978), [3]H_{obs} = Observed heterozygosity = No. of heterozygotes / N, [4]E_5a = Allelic evenness

Multilocus genotypic diversity

Each plot harboured a genotypically diverse population. On average, we detected 9.5 genotypes out of every 14 isolates sampled, showing that 67% of the isolates belonged to a unique genotype (Table 5.4). Genotypic diversity (*H*, *G* and λ) and richness (*eMLL*) were

highly variable, ranging from very low (H = 0.97, G = 2.46, λ = 0.59, *eMLL* = 3) for a plot with only three MLLs (LSiiT2P3) to very high (H = 2.76, G = 14.29, λ = 0.93, *eMLL* = 7) for a population where 17 out of 20 individuals sampled belonged to different MLLs (DST1P3). Total average genotypic diversity (H = 2.02, G = 7.54, λ = 0.8) was low, and total average richness was high (*eMLL* = 5.5). Evenness (E_5b) ranged from 0.62 to 1.0 and averaged 0.86, showing that genotypes were evenly distributed. Genetic diversity ($\hat{h}$) for subpopulations ranged from 0.34 to 0.64 and averaged 0.48 over all samples. The clonal fraction ranged from 0.00 to 0.67 (Table 5.4).

Each of the genotypic diversity indices, H, G and λ, decreased markedly within populations across rotation, beginning with moderate to high averages of 2.45, 11.27, 0.90, respectively, across 1st rotation populations, decreasing sharply to 1.81, 6.14, 0.8 in 2nd rotation populations, with a further, yet less dramatic decrease to averages of 1.81, 5.54 and 0.78 across 3rd rotation populations (Table 5.4). Similarly, genotypic evenness declined from almost equal genotype abundance ($E_5\,b$ = 0.93) in 1st rotation plots, to 0.89 in 2nd and 0.78 in 3rd rotation populations. Genetic diversity ($\hat{h}$) varied from 0.34 to 0.69 and there was no evident trend for subpopulations across rotation (Table 5.4). The average clonal fraction was low in 1st rotation plots at *CF* = 0.12, increasing sharply to 0.38 across 2nd rotation populations, with a smaller increase to an average of 0.40 across 3rd rotation populations (Table 5.4).

Genotypic richness (*eMLL*, as calculated by rarefaction) and, genotypic diversity (G) and evenness (E_5c) were all lower in the second and third rotation populations from second (Logas South 2 and Langgam) and third (Baserah and Sebulu) rotation plots(Logas South 2, Langgam, Baserah and Sebulu) and evenness (E_5c) also declined with each successive rotation (Table 5.5).

Table 5.4. Site, plot, sample sizes, and number of genotypes observed in six populations of *Ganoderma philippii*, ranging from 1st to 3rd rotation *Acacia* plantations.

Site[a]	Plot[a]	*N*[b]	*MLL*[c]	*eMLL*[d]	*SE*[e]	*H* (95% CI)[f]	*G* (95% CI)[g]	*λ* (95% CI)[h]	E_5b (95% CI)[i]	$\hat{h}$[j]	*CF*[k]
Rotation 1											
Deras	DST1P1	8	7	6	0.00	1.91 (1.5-2.31)	6.4 (4.66-8.14)	0.84 (0.72-0.97)	0.94 (0.81-1.07)	0.53	0.13
Deras	DST1P3	20	17	7	0.76	2.76 (2.47-3.06)	14.29 (11.16-17.41)	0.93 (0.88-0.98)	0.9 (0.78-1.01)	0.41	0.15
Deras	DST1P4	10	10	7	0.00	2.3 (1.95-2.65)	10 (7.91-12.09)	0.9 (0.82-0.98)	1 (0.9-1.1)	0.57	0.00
Logas South 1	LSiT1P1	21	16	6	0.90	2.66 (2.37-2.96)	12.6 (9.67-15.53)	0.92 (0.87-0.97)	0.87 (0.75-0.98)	0.44	0.24
Logas South 1	LSiT1P3	11	11	7	0.00	2.4 (2.08-2.72)	11 (8.88-13.12)	0.91 (0.84-0.97)	1 (0.91-1.09)	0.59	0.00
Logas South 1	LSiT1P4	20	16	6	0.82	2.69 (2.41-2.98)	13.33 (10.47-16.19)	0.93 (0.88-0.97)	0.9 (0.78-1.01)	0.48	0.20
Average		15.00	12.83	6.5	0.41	2.45 (2.13-2.78)	11.27 (8.79-13.75)	0.90 (0.84-0.97)	0.93 (0.82-1.05)	0.50	0.12
Rotation 2											
Langgam	LGT1P1	24	15	6	1.02	2.54 (2.23-2.85)	10.67 (7.75-13.58)	0.91 (0.85-0.96)	0.83 (0.7-0.95)	0.37	0.38
Langgam	LGT1P2	11	7	5	0.50	1.77 (1.32-2.22)	4.84 (2.97-6.71)	0.79 (0.63-0.95)	0.79 (0.61-0.97)	0.41	0.36
Langgam	LGT1P3	13	9	6	0.75	2.1 (1.73-2.47)	7.35 (5.25-9.45)	0.86 (0.77-0.96)	0.89 (0.75-1.02)	0.49	0.31
Logas South 2	LSiiT1P1	7	7	7	0.00	1.95 (1.53-2.36)	7 (5.27-8.73)	0.86 (0.73-0.98)	1 (0.89-1.11)	0.46	0.00
Logas South 2	LSiiT1P5	12	5	4	0.39	1.55 (1.24-1.85)	4.5 (3.35-5.65)	0.78 (0.67-0.89)	0.95 (0.82-1.08)	0.35	0.58
Logas South 2	LSiiT2P3	8	3	3	0.00	0.97 (0.59-1.36)	2.46 (1.61-3.31)	0.59 (0.38-0.8)	0.89 (NA-NA)	0.5	0.63
Average		12.50	7.67	5.2	0.44	1.81 (1.44-2.19)	6.14 (4.37-7.9)	0.8 (0.67-0.75)	0.89 (0.75-1.03)	0.43	0.38
Rotation 3											
Baserah	BST2P1	27	9	4	1.08	1.7 (1.33-2.08)	4.07 (2.55-5.59)	0.75 (0.64-0.86)	0.68 (0.56-0.81)	0.43	0.67
Baserah	BST2P2	10	5	4	0.00	1.23 (0.63-1.83)	2.5 (1-4)	0.6 (0.3-0.9)	0.62 (NA-NA)	0.34	0.50
Baserah	BST2P6	14	10	6	0.83	2.14 (1.72-2.56)	7 (4.53-9.47)	0.86 (0.74-0.97)	0.8 (0.63-0.96)	0.5	0.29
Sebulu	SBT1P1	13	11	6	0.64	2.35 (2.02-2.68)	9.94 (7.72-12.16)	0.9 (0.83-0.97)	0.94 (0.83-1.05)	0.64	0.15
Sebulu	SBT1P2	13	5	4	0.67	1.31 (0.89-1.73)	3.07 (1.88-4.26)	0.67 (0.5-0.85)	0.76 (0.59-0.94)	0.49	0.62
Sebulu	SBT1P3	9	7	6	0.00	1.83 (1.38-2.29)	5.4 (3.53-7.27)	0.81 (0.66-0.97)	0.84 (0.68-1)	0.53	0.22
Sebulu	SBT1P4	15	10	6	0.92	2.12 (1.72-2.52)	6.82 (4.46-9.18)	0.85 (0.75-0.96)	0.79 (0.65-0.94)	0.57	0.33
Average		14.43	8.14	5.1	0.59	1.81 (1.38-2.24)	5.54 (3.67-7.42)	0.78 (0.63-0.93)	0.78 (0.65-0.95)	0.50	0.40
Total (pooled)		266	168	7	0.49	2.02 (1.64-2.39)	7.54 (5.51-9.6)	0.8 (0.71-0.94)	0.86 (0.74-1.00)	0.48	0.30

[a]In all, 3 to 4 plots (referred to as subpopulations) of 10 x 10 trees were sampled, from six plantation stands (referred to as sites or populations) that had a history of *Ganoderma* root rot. Plots are physically separated by 300-700m, and the six site locations are physically separated by ≥15kms as the crow flies. [b]***N*** = Sample size. [c]***MLL*** = Number of unique multilocus lineages observed, is equivalent to genotypic richness. [d]***eMLL*** = Number of expected MLLs based on rarefaction at smallest sample size N = 7. [e]***SE*** = Standard error of rarefaction analysis. [f]***H*** = **S**hannon-Wiener Index of MLL diversity. [g]***G*** = Stoddart and Taylor's Index of MLL diversity. [h]λ = Simpson's index. [i]E_5b = Evenness. [j]$\hat{h}$ = Nei's genetic diversity, also referred to as Nei's expected heterozygosity**.** [k]***CF*** = Clonal Fraction (1 - (MLL/N)).

Table 5.5. Population genotypic diversity measures for populations by site, including all individuals or corrected for clones occurring within each plot.

					Complete data					Clone-corrected analysis				
Site (population)	***N*[a]**	***MLL*[b]**	***eMLL*[c]**	***SE*[d]**	***G*[e] (95% CI)**	***E_5c*[f] (95% CI)**	***$\hat{h}$*[g]**	***H_{obs}*[h]**	***PTLPT*[i]**	***N***	***$\hat{h}$***	***H_{obs}***	***PTLPT***	***F_{ST}*[j]**
Deras	38	34	25	0.95	30.08 (25.78-34.38)	0.93 (0.85-1.01)	0.55	0.29	<0.001	34	0.57	0.31	<0.001	0.51
Logas South 1	52	37	22	1.49	28.77 (24.04-33.49)	0.87 (0.79-0.95)	0.51	0.35	<0.001	43	0.54	0.4	<0.001	0.29
Langgam	48	29	20	1.54	21.33 (17.38-25.28)	0.85 (0.76-0.94)	0.45	0.34	<0.001	31	0.47	0.39	<0.001	0.21
Logas South 2	27	13	13	0.00	8.19 (5.85-10.53)	0.79 (0.66-0.92)	0.46	0.53	<0.001	15	0.49	0.51	<0.001	0.06
Baserah	51	23	15	1.68	10.04 (6.85-13.23)	0.66 (0.56-0.77)	0.49	0.37	<0.001	24	0.56	0.45	<0.001	0.32
Sebulu	50	32	20	1.63	20.49 (15.87-25.11)	0.78 (0.67-0.89)	0.63	0.54	<0.001	33	0.65	0.58	<0.001	0.17
Total (pooled)	266	168	25	1.34	98.27 (86.42-110.12)	0.74 (0.67-0.8)	0.59	0.40	<0.001	180	0.61	0.44	<0.001	0.26

[a]***N*** = Sample size. [b]***MLL*** = Number of unique multilocus lineages observed. [c]***eMLL*** = Number of expected MLLs based on rarefaction at smallest N =27. [d]***SE*** = Standard error of rarefaction analysis. [e]***G*** = Stoddart and Taylor's Index of MLL diversity. [f]***E_5c*** = Evenness. [g]***$\hat{h}$*** = Nei's genetic diversity, also referred to as Nei's expected heterozygosity**.** [h]***H_{obs}*** = Observed heterozygosity. [i]***PTLPT*** = parsimony tree length permutation test, where significance level of PTLPT is based on comparison of length of observed parsimony tree to lengths of trees generated from 1,000 permuted data sets (Burt *et al.* 1996). [j]***F_{ST}*** = the proportion of the total genetic variance contained in a subpopulation relative to the total genetic variance.

Distribution of multilocus lineages

Of the 168 MLLs, 125 were unique to a single isolate, pairs of isolates in the same MLL were recovered 19 times, there were 10 groups of 3, nine groups of 4, two groups of 6, and the remaining isolates fell into single groups of seven, eight, and ten isolates assigned to the same MLLs (Figure 5.2). The majority of these groups occurred within the same plot at the same site. MLL.63 and MLL.92 were the most abundant genotypes (n=10 and n=8 respectively), and were both recovered from a single 3rd rotation plot at the Baserah Site. Only 11 MLLs were shared among neighbouring plots and no MLLs were shared among sites (Figure 5.2).

Thirteen individuals shared five MLLs among first rotation plots, while 15 individuals shared four MLLs among second rotation plots and nine individuals shared two MLLs among third rotation plots (Figure 5.2). Within plots, clones were spatially clustered (Figure S5.6).

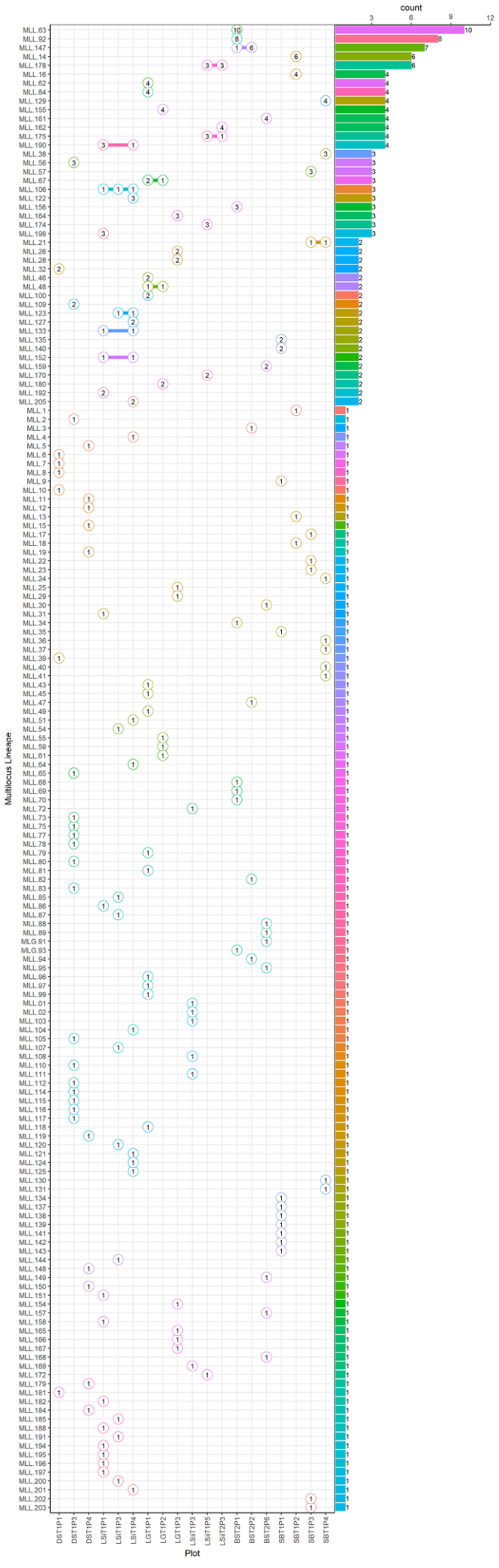

Figure 5.2. Rank distribution of multilocus lineages (MLLs) of *Ganoderma philippii* and recovery per plot. The vertical axis denotes unique MLLs detected in the whole data set with decreasing abundance, as indicated by the bar plot on the right side. The horizontal axis indicates sampling plot. Each numbered circle represents the number of observations of each MLL, with lines connecting MLLs found in multiple plots.

Selfing and clonality in populations

PTLPT

Analysis based on PTLPT revealed a tree length for the observed data that was significantly shorter than expected for freely recombining organisms for both total and clone-corrected data sets ($P < 0.001$) (Table 5.5).

Index of multilocus disequilibrium

Despite the high genetic diversity, some level of clonal reproduction occurred in the majority of plots. Based on the complete data set, the null hypothesis of HWE was rejected in most sub-populations (plots) for which a sufficient number of isolates was obtained, the exceptions included two first rotation and two second rotation plots (Table 5.6). More exceptions were obtained from the clone-corrected analysis, with two plots from each rotation appearing to be at HWE. When populations defined by site of origin were analysed, the $\bar{r}d$ test rejected the hypothesis of HWE for all populations ($P < 0.01$) based on both the complete and clone-corrected datasets (Table 5.6).

Table 5.6. Multilocus linkage disequilibrium ($\bar{r}d$) within *Ganoderma philippii* sub-populations (plots) and populations (sites) at 1st, 2nd and 3rd rotation. A value significantly different (P <0.01) from zero indicates that the population is not at Hardy-Weinberg equilibrium. Tests were performed on all isolates and on clone-corrected data but calculations based on sample sizes of <10 individuals are deemed unreliable (nc).

Populations		**All isolates**		**Clone-corrected**	
Site	**Plot**	$\bar{r}d$	***P* value**	$\bar{r}d$	***P* value**
Rotation 1					
Deras	DST1P1	nc	nc	nc	nc
Deras	DST1P3	0.062	0.013	0.025	0.138
Deras	DST1P4	0.009	0.344	0.009	0.357
Logas South1	LSiT1P1	0.254	<0.001	0.191	<0.001
Logas South1	LSiT1P3	0.084	0.011	0.084	0.013
Logas South1	LSiT1P4	0.253	<0.001	0.201	<0.001
Rotation 2					
Langgam	LGT1P1	0.113	<0.001	0.056	0.032
Langgam	LGT1P2	0.215	<0.001	nc	nc
Langgam	LGT1P3	0.255	<0.001	0.223	<0.001
Logas South2	LSiiT1P3	nc	nc	nc	nc
Logas South2	LSiiT1P5	0.050	0.075	nc	nc
Logas South2	LSiiT2P3	nc	nc	nc	nc
Rotation 3					
Baserah	BST2P1	0.264	<0.001	0.069	0.027
Baserah	BST2P2	0.239	<0.001	0.017	0.311
Baserah	BST2P6	0.219	<0.001	0.117	0.002
Sebulu	SBT1P1	0.160	<0.001	0.111	<0.001
Sebulu	SBT1P2	0.425	<0.001	nc	nc
Sebulu	SBT1P3	0.434	<0.001	nc	nc
Sebulu	SBT1P4	0.381	<0.001	0.239	<0.001
Deras	N = 3	0.06	<0.001	0.03	0.009
Logas South 1	N = 3	0.18	<0.001	0.15	<0.001
Langgam	N = 3	0.09	<0.001	0.08	<0.001
Logas South 2	N = 3	0.23	<0.001	0.13	<0.001
Baserah	N = 3	0.14	<0.001	0.06	0.002
Sebulu	N = 4	0.14	<0.001	0.08	<0.001
	Total	0.04	0.00	0.03	0.001

5.3.2. Spatial population structure of *Ganoderma philippii*

Principal co-ordinates analysis revealed no clear trend in the geographic structure of the data (Figure 5.3). The Bayesian Information Criterion (Figure S5.4) was ambiguous, indicating that 11-20 clusters would provide useful summaries of our data (Jombart and Collins 2017), hence we only proceeded with the second DAPC method: using i) 19 plot sampling localities and ii) six site sampling localities, as priors (Jombart *et al.* 2010).

DAPC analysis revealed low genetic differentiation between plots (Figure S5.5), sites and region (Figure 5.4). As no genetic structure had been detected among the isolates across the two sampled regions (with a distance of 1550 km between the most distant collection points), we ran a Mantel test to search for a potential isolation-by-distance phenomenon. This test revealed a significant difference between simulated data and observed data after permutations (correlation coefficient = 0.227347, P = 0.0001), indicating a weak positive correlation between genetic and geographic distance (so that only about 5.1% (*i.e.* 0.227347^2) of the genetic divergence is explained by geographic distance). As there was no noticeable difference between samples from Kalimantan and Sumatra, we then searched for a smaller sampling scale that might highlight a population structure. For this we used hierarchical analyses of molecular variance (AMOVA).

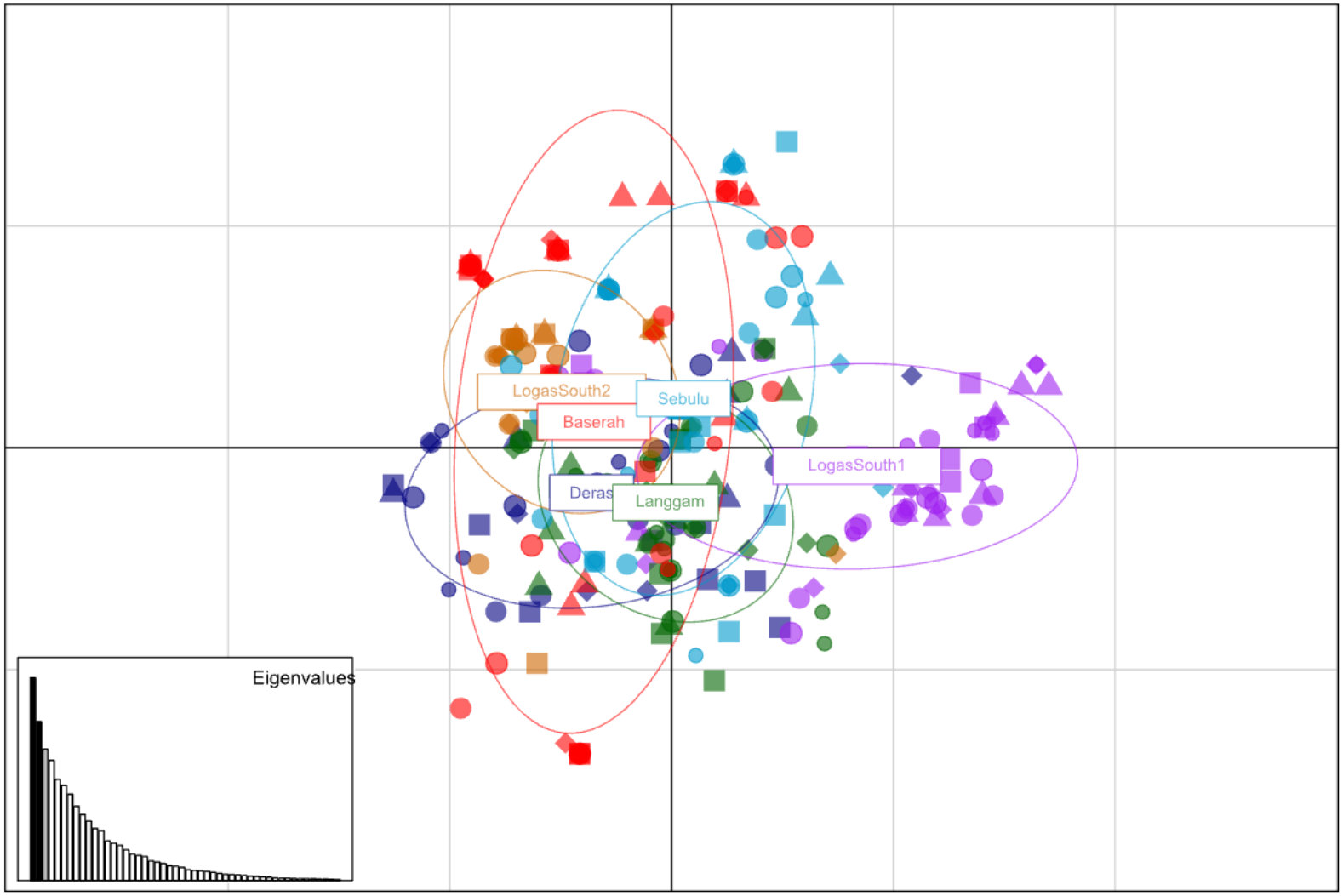

Figure 5.3. Global genetic diversity observed by Principle Co-ordinates Analysis (PCA) analysis of the first two principal components discriminating *Ganoderma philippii* populations by regions based on genotyped samples without missing data and using nine microsatellite loci considered without null alleles. Points represent individual observations, with their colours representing site membership and their shapes representing plot. Inertia ellipses represent an analogue of a 67% confidence interval based on a bivariate normal distribution.

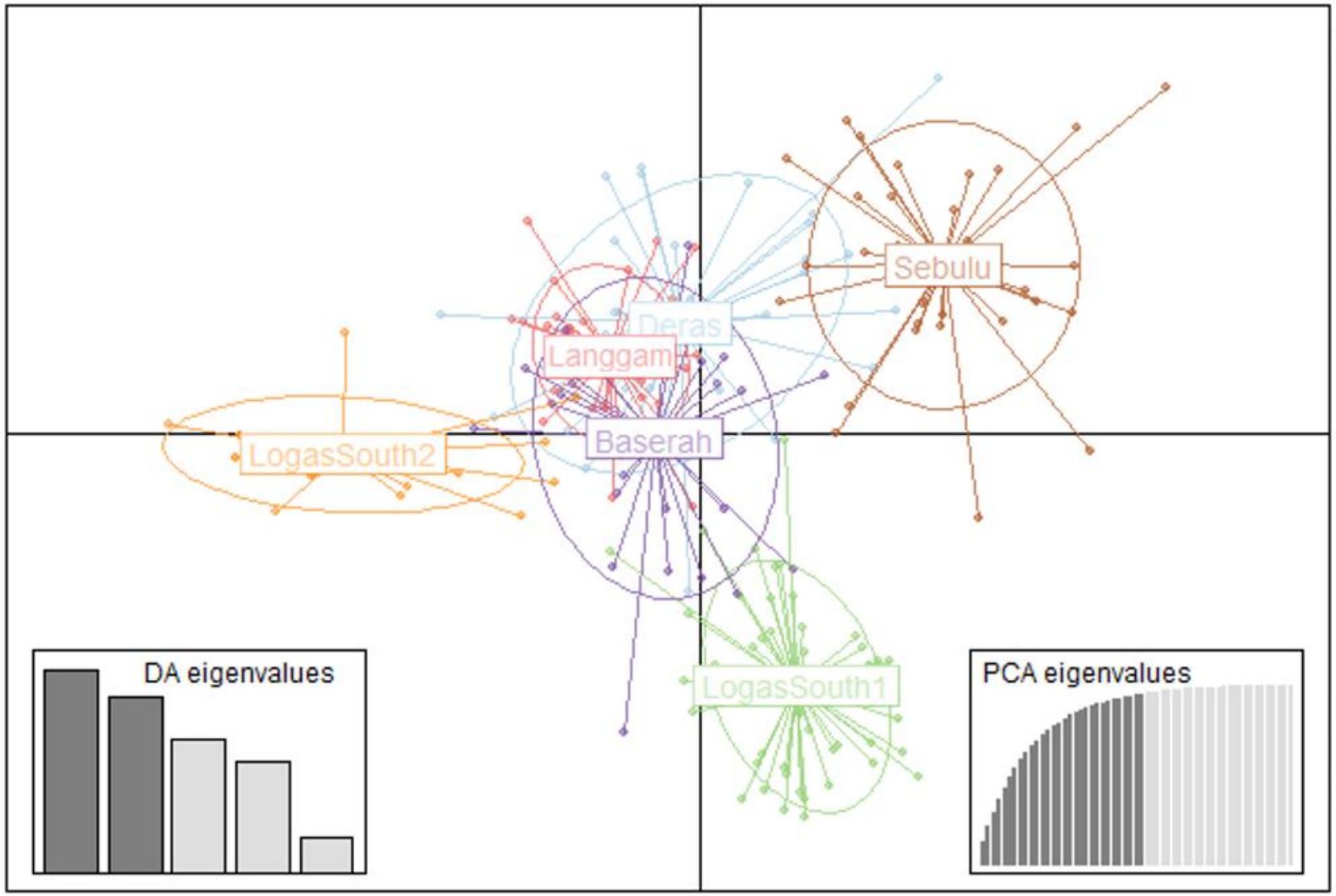

Figure 5.4. Scatterplot from discriminant analysis of principal components (DAPC) of the first two principal components discriminating *Ganoderma philippii* populations by regions (site location given as a priori). Points represent individual observations. Lines and shapes represent population membership. Inertia ellipses represent an analogue of a 67% confidence interval based on a bivariate normal distribution.

Populations of *G. philippii* were significantly differentiated at the site (among populations), plot (within populations and among subpopulations), and isolate level for both the complete dataset and the clone corrected data ($P < 0.001$) (Table 5.7). A total of 65% of the variance was associated with differences among sites and 22% with differences among sub-populations (plots) within populations, whereas only 12 % of the variance was attributable to variation among individuals within subpopulations (Table 5.7). Fixation indices were all low but were lowest within subpopulations (the isolate level Table 5.7). Comparative analysis of the clone-corrected and total population resulted in qualitatively similar results. Gene diversity, genetic distance, and population differentiation remained qualitatively similar in the clone-corrected analysis (Tables 5.4, 5.5 and 5.7). A minimum spanning network of genotypes (Figure 5.5) also illustrates the distinction among sites but, in addition, shows some potential migrants.

Table 5.7. Analysis of molecular variance for microsatellite data of six *Ganoderma philippii* populations in Deras, Logas South 1, Langgam, Logas South 2, Baserah and Sebulu. Variance was partitioned among and within six populations and within 3 (Deras, Logas South 1, Langgam, Logas South 2, Baserah) or 4 (Sebulu) subpopulations.

Hierarchical level	**Variation (%)**	**Φ**	**P**
Analysis of all individuals			
ΦCT (among populations - site)	65.5	0.34	0.001[a]
ΦSC (among subpopulations within populations - plot)	22.2	0.25	0.001[b]
ΦST (within subpopulations - isolate)	12.2	0.12	0.001[c]
Clone-corrected analysis			
ΦCT (among populations - site)	74.8	0.25	0.001[a]
ΦSC (among subpopulations within populations - plot)	13.6	0.15	0.001[b]
ΦST (within subpopulations isolate)	11.6	0.12	0.001[c]

[a]Probability of obtaining equal or lower Φ value determined by 1,000 randomizations by permuting subpopulations among populations.
[b]Probability of obtaining equal or lower Φ value determined by 1,000 randomizations by permuting genotypes among subpopulations within populations.
[c]Probability of obtaining equal or lower Φ value determined by 1,000 randomizations by permuting genotypes among subpopulations among population.

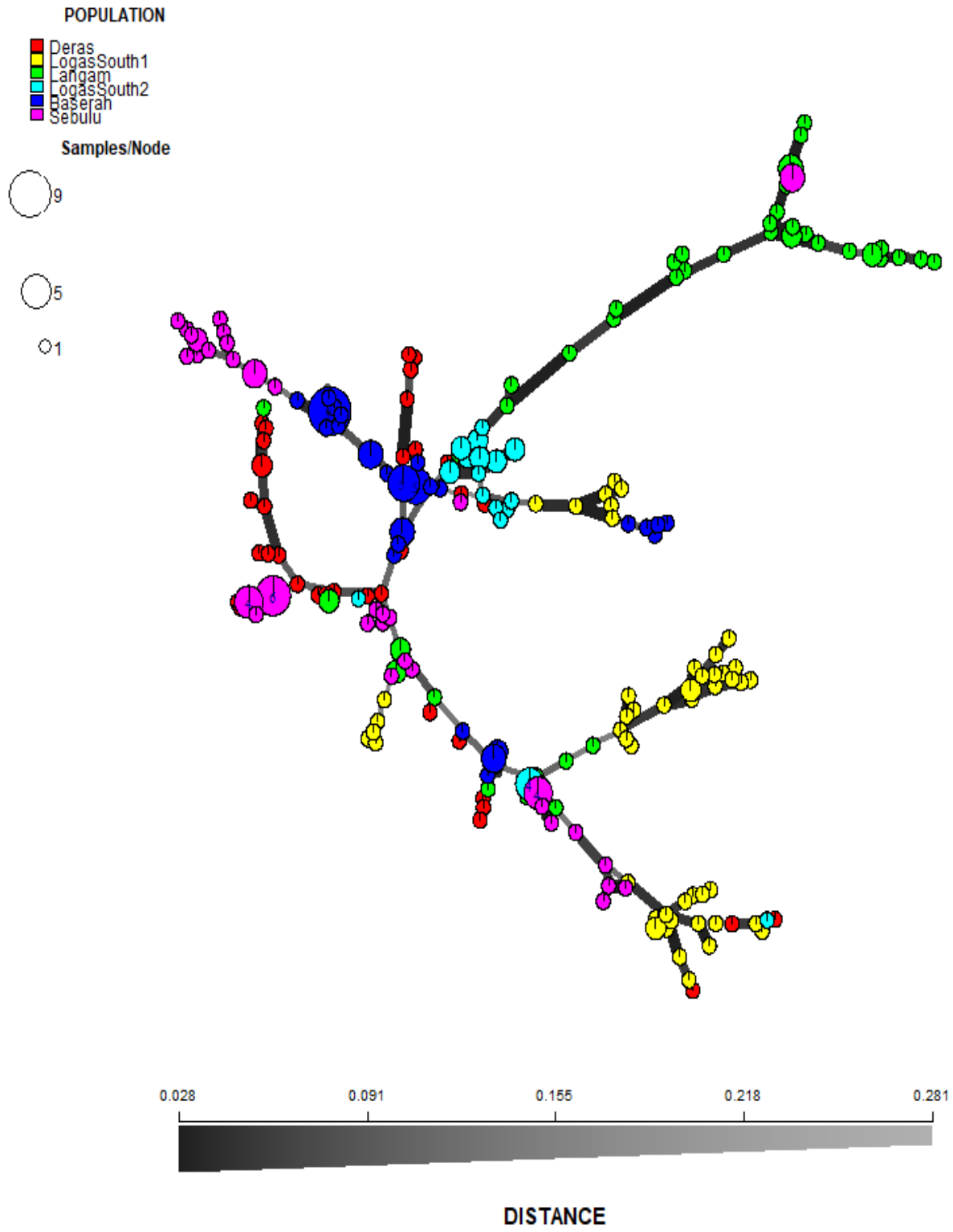

Figure 5.5. Minimum spanning network based on Bruvo's genetic distance for microsatellite markers for *Ganoderma philippii* populations. Nodes (circles) represent individual multilocus genotypes. Node colours/shades represent population membership proportional to the pie size. Node sizes are relatively scaled to $\log_{1.75}n$, where n is the number of samples in the nodes to avoid node overlap. Lines represent minimum genetic distance between individuals determined by Prim's algorithm. Nodes that are more closely related will have darker and thicker lines whereas nodes more distantly related will have lighter and thinner lines or no line at all. Reticulation was introduced by finding exact ties in genetic distance after Prim's algorithm was run.

5.4. Discussion

Clonal fraction and size of individual clones (in terms of number of trees colonised) increased after the first rotation. The effect on genetic diversity was insignificant, with Mantel Spatial tests indicating that overall only 5.1% of genetic differentiation was influenced by clonal dispersal. Clones in first rotation plots were more widely dispersed than in second and third rotation plots; of the few MLLs that occurred in different plots at the same location, half were in first-rotation plots at Logas South 1, with one clone extending to all three plots. Assuming that the previous vegetation was rainforest, the hosts were most likely large trees with extensive root systems, presumably co-evolved and with greater tolerance to colonisation by *G. philippii*. After the felling of rainforest trees, woody inoculum would have been present at the time of plantation establishment with *A. mangium*, a naïve host with low tolerance to *G. philippii* (Francis *et al.* 2014). The close spacing of trees in *A. mangium* plantations (2 x 3m) and the high susceptibility to root rot would have facilitated clonal spread to adjacent trees by root contact; in second and third rotation plots, identical MLLs were frequently isolated from contiguous rows of trees. Root to root spread has previously been implicated in the spread of *Ganoderma* root rot in *Acacia* plantations in Indonesia, based on spatial analysis (Francis *et al.* 2014) and is a common strategy of many basidiomycete root rot pathogens.

Several analyses indicate that *G. philippii* populations in *Acacia* plantations are highly inbred, even when clone-corrected data is analysed. These indications include a lack of HWE equilibrium at population and sub-population levels with heterozygosity significantly lower than expected at all sampling scales. Using clone-corrected data, observed heterozygosity in some of the plots (sub-populations) was not significantly different from expected, however when populations were grouped at the site level, observed heterozygosity was well below the expected. Additionally, the PTLPT tree length is shorter than expected and the highest proportion of the variance, 65%, is among populations (sites) and the next, 22%, among sub-

populations (plots) with only 12% within plots. This contrasts strongly with the lack of structure in *G. boninense* populations, in which 97% of the variation was within plantations, 1% was between plantations and 1.6% between regions (Mercière *et al.* 2017).

These results all support rejections of the hypothesis of random mating and lend support to the presence of a mixed reproductive system, including selfing and occasional out-crossing. *Ganoderma philippii* has a tetra polar mating system, favouring out-crossing (Page *et al.* 2018) however other factors which increase the likelihood of sibling mating may predominate. *G. philippii* sporocarps frequently occur low on the tree trunk, often hidden by undergrowth, a situation from which it would be more difficult for spores to become air-borne, in contrast to basidiocarps of *G. australe* and *G. mastoporum* which may occur higher on the trunk. Additionally, basidiospores are produced in lower abundance by *G. philippii* than by *G. australe* or *G. mastoporum* and are fastidious in their requirements for germination (Lim 1977; Page *et al.* 2017). Genetic diversity remained high at all sampling scales, irrespective of rotation, plot, site or region. Nevertheless, it is unclear whether populations in second and third rotation plantations establish mainly from persistence of woody inoculum or establishment of new infections from basidiospores. While the greatest genetic variation was detected between populations (sites), genetic distance is not correlated with geographic distance. This may be explained by largely in-breeding populations with occasional immigrants from further afield and this explanation is supported by the minimum spanning network, which shows clusters of very closely related individuals at each site with scattered individuals from one location that are more closely linked genetically to a different population.

The Sebulu population, which is on a different island, is no more genetically distinct from the Sumatran populations than they are from each other, indicating that a distance of 1550 km and an intervening sea present no greater barrier to genetic exchange than a distance of one to six kilometres. Air currents, insects or even humans may all play a role in assisting spore

dispersal between islands. Basidiospores of *G. philippii* remain viable after being eaten and excreted by tipulid flies in the genus *Limonia* (Lim 1977, as *G. pseudoferreum*) and this could provide a means for small numbers of basidiospores to travel longer distances.

Basidiospores have been implicated as the main inoculum source for *G. boninense*, causal agent of basal stem rot (BSR) in oil palms (Mercière *et al.* 2017; Pilotti *et al.* 2003) and infection mechanisms for basidiospores have been elucidated (Rees *et al.* 2011). The population structure of *G. boninense* contrasts strongly with that of *G. philippii*, showing little differentiation among populations across northern Sumatra and Peninsular Malaysia (Mercière *et al.* 2017) and apparently comprised of one large, freely recombining population, with little clonal duplication. Early work with *G. boninense* focussed on the potential for root to root spread of the pathogen and the sanitation procedures adopted may have reduced the level of clonal propagation and influenced the population structure of this pathogen (Mercière *et al.* 2017).

The data presented here for *G. philippii*, showing that there is considerable genotypic variation even at the plot level, coupled with significant population sub-structuring, provide circumstantial evidence that out-crossing and migration occur, albeit rarely, and contribute to genetic diversity and population differentiation. It appears that the model best describing the population structure of *G. philippii* is a variation of the epidemic model discussed by Maynard Smith et al. (1993), where sexual recombination predominantly between sibling basidiospores results in the linkage associations observed, but with a low migration rate providing for allele richness. This is similar to a population structure due to mixed modes of reproduction or mating that include clonal (vegetative) propagation and re-combination, both in-breeding and out-crossing, as observed for other fungi (Billiard *et al.* 2012; Laflamme 2010; Woodward *et al.* 1998).

Knowledge of a pathogen's mode of reproduction has implications for disease management, as exemplified by a related pathogen, *G. boninense,* which causes basal stem rot in oil palms in Malaysia and PNG. Studies based on mitochondrial DNA markers, mating alleles, somatic (or vegetative) compatibility and microsatellites have all revealed high levels of genetic diversity (Mercière *et al.* 2017; Pilotti *et al.* 2003). Infections by separate genotypes must have arisen through sexual recombination and subsequent dispersal via spread of basidiospores. Understanding the biology of *G. boninense* at these levels has informed changes in disease management (Mercière *et al.* 2017).

The use of a basidiomycete biological control agent to prevent the colonisation of harvested stumps was pioneered using *Phlebiopsis gigantea* (Fr.) Jülich, against *Heterobasidion annosum* (Fr.) Bref. (Pratt 1998) and a similar strategy has been proposed for the *G. philippii/A. mangium* pathosystem (Agustini *et al.* 2014b). This may be an effective strategy to prevent new infections initiated by basidiospores in disease-free areas, but given the propensity for clonal spread, is not expected to eradicate the pathogen from plantations with pre-existing inoculum. As mechanical stump removal is relatively expensive and may not to remove all infected roots, additional strategies to manage woody inoculum may be required.

5.5. Supplementary Material

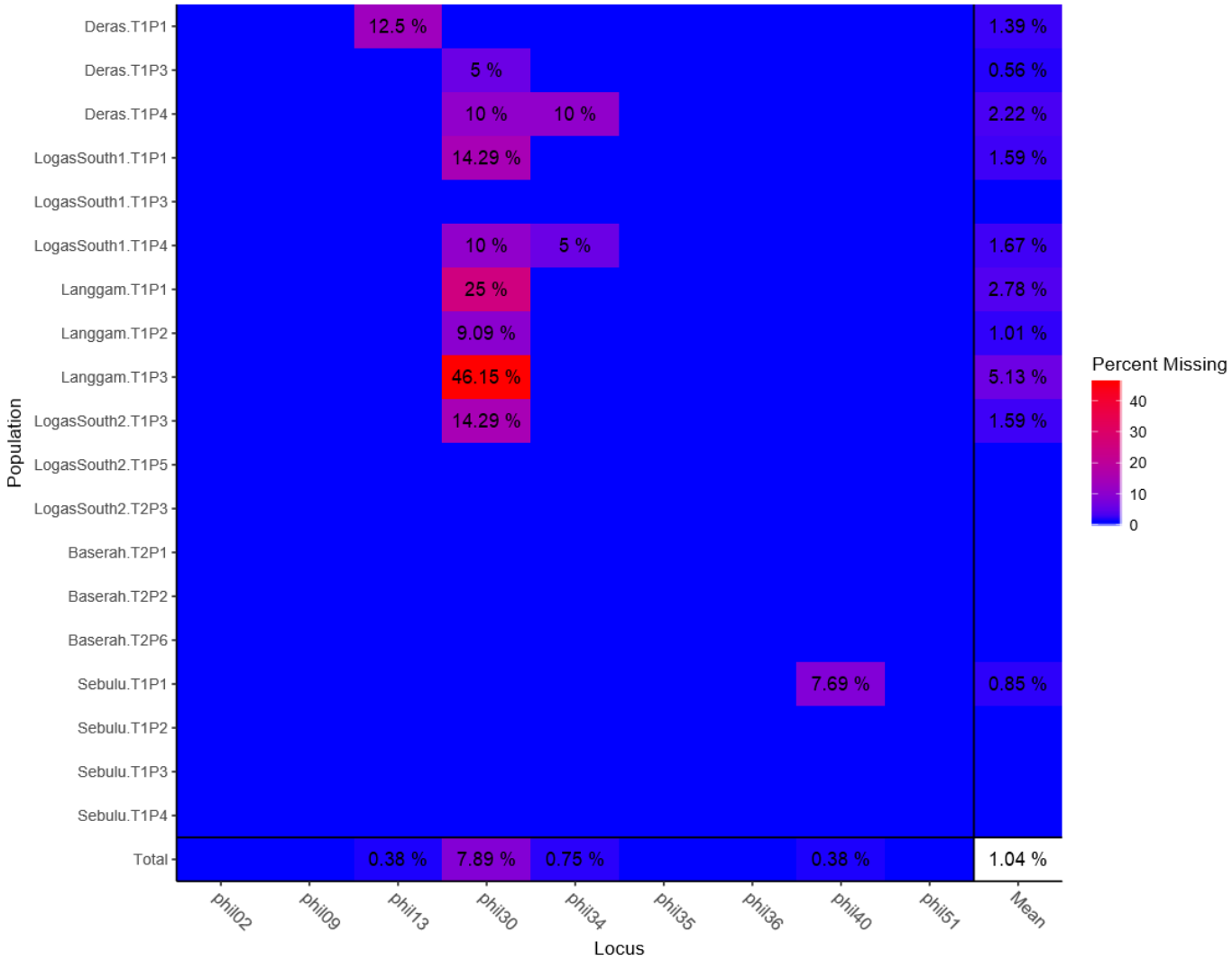

Figure S5.1. Summary of percent missing data per locus and per site by plot.

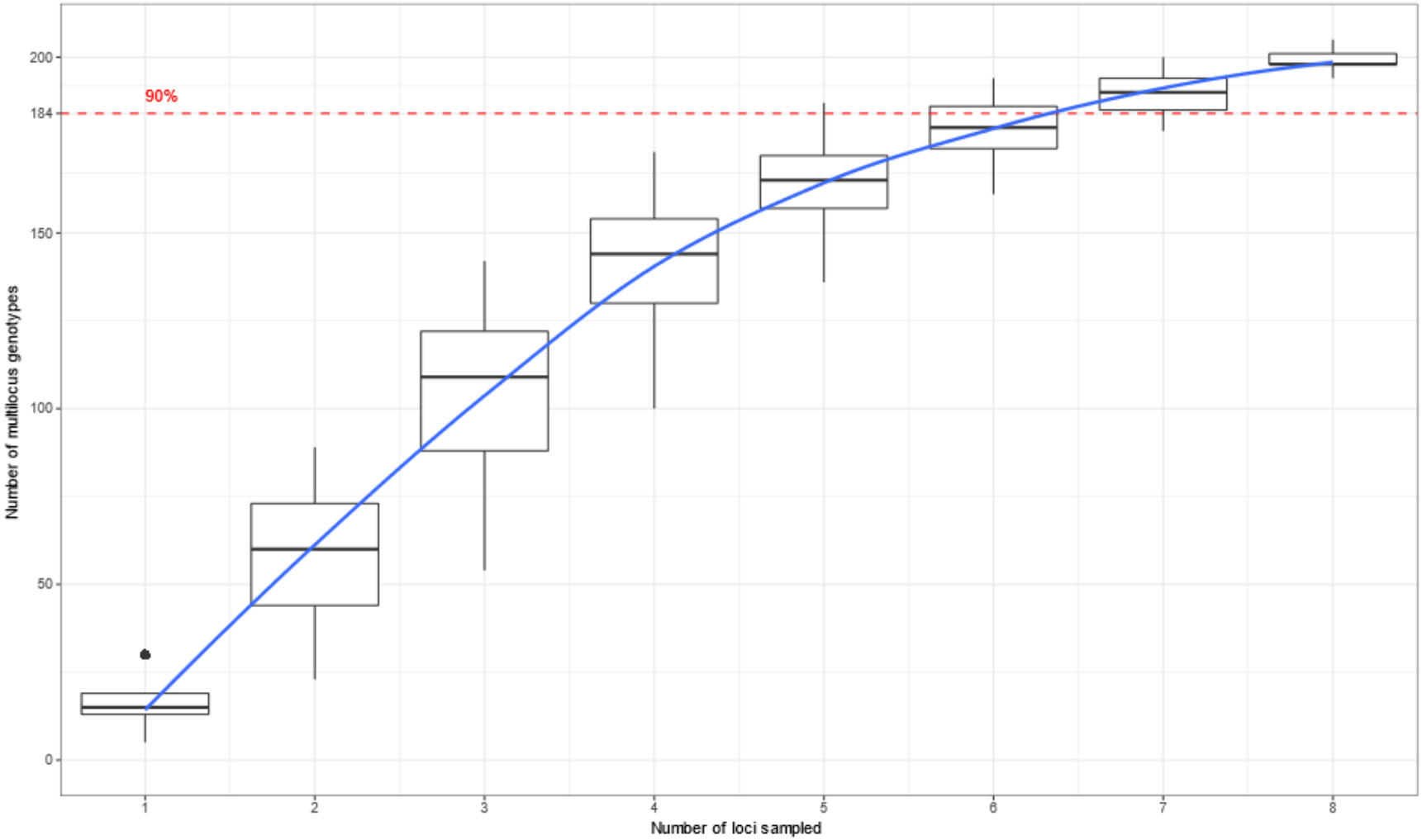

Figure S5.2. Genotype accumulation curve for 266 isolates of *G. philippii* genotyped over 9 loci. The horizontal axis represents the number of loci randomly sampled without replacement up to n − 1 loci, the vertical axis shows the number of multilocus genotypes (MLGs) observed, up to 205, the number of unique MLGs in the data set (defined by naïve string comparison). The red dashed line represents 90% of the total observed MLGs.

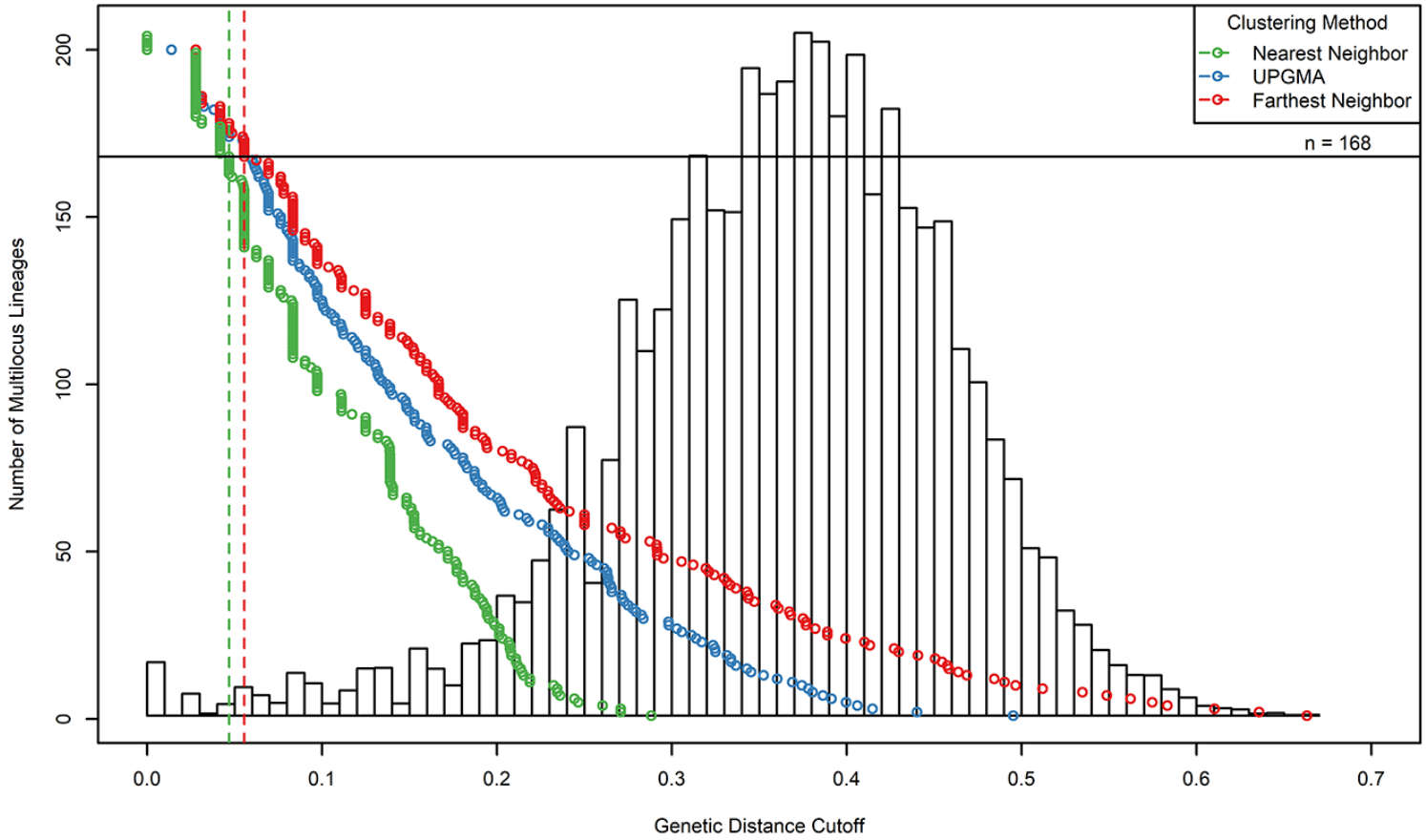

Figure S5.3. Graphical representation of three different clustering algorithms assessed for collapsing 205 *G. philippii* multilocus genotypes (MLGs) for 9 SSR loci representing 168 multilocus lineages (MLLs). The horizontal axis is Bruvo's genetic distance assuming the genome addition model. The vertical axis represents the number of MLLs observed. Each point shows the threshold at which one would observe a given number of MLGs. The horizontal black line represents 168 MLLs (our expected number of MLLs at the set threshold of 0.056) and the vertical dashed lines mark the various thresholds used to collapse the MLGs into 168 MLLs for each algorithm

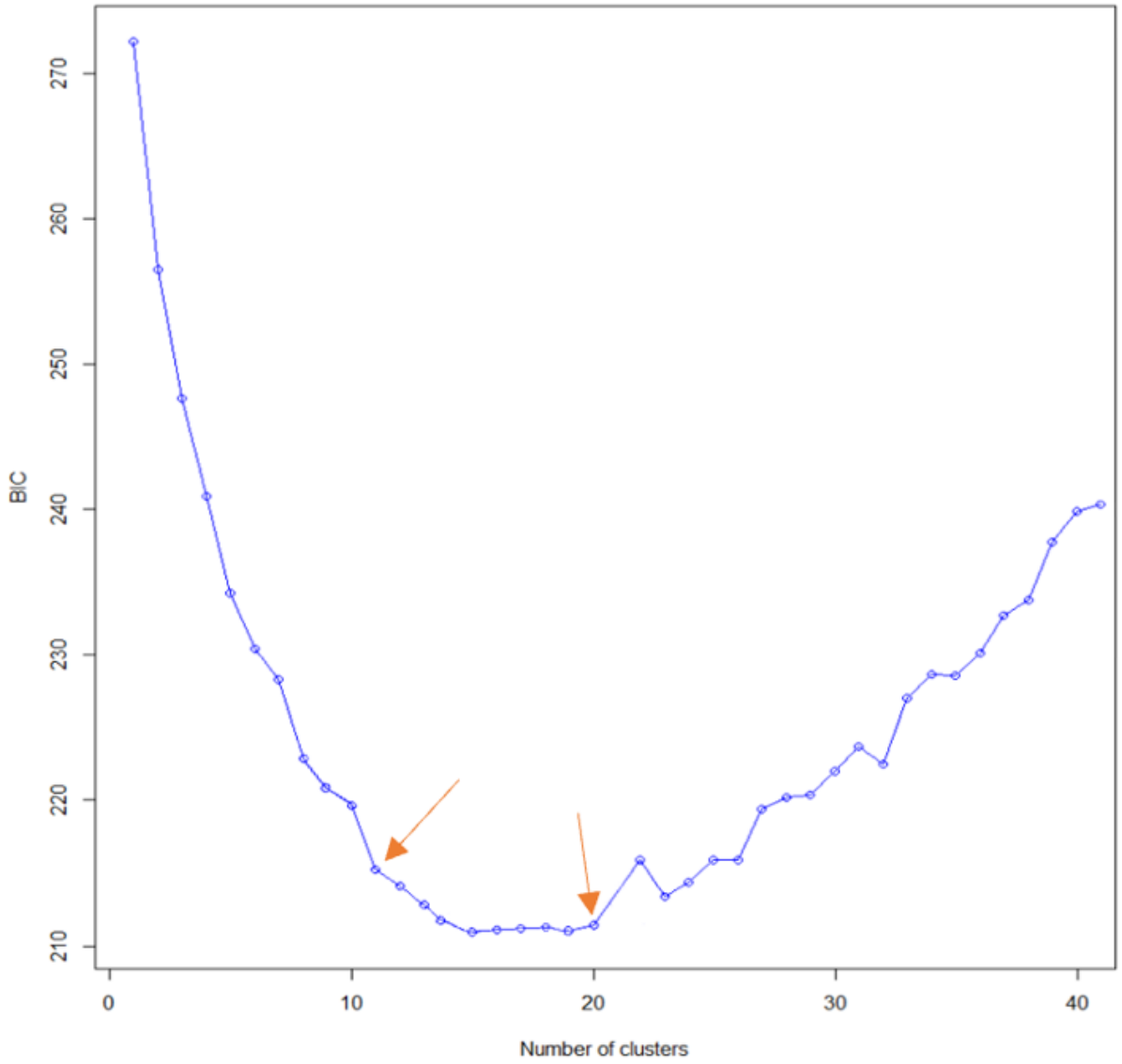

Figure S5.4. Results of K-means clustering method *find.clusters*, used to identify the number of clusters with each clustering solution labeled from K = 1 to 40, and its corresponding Bayesian information criterion (BIC) score. The lowest BIC score corresponded to K = 15, however the BIC decreases observed indicate that K=11-20 would provide useful summaries of our data.

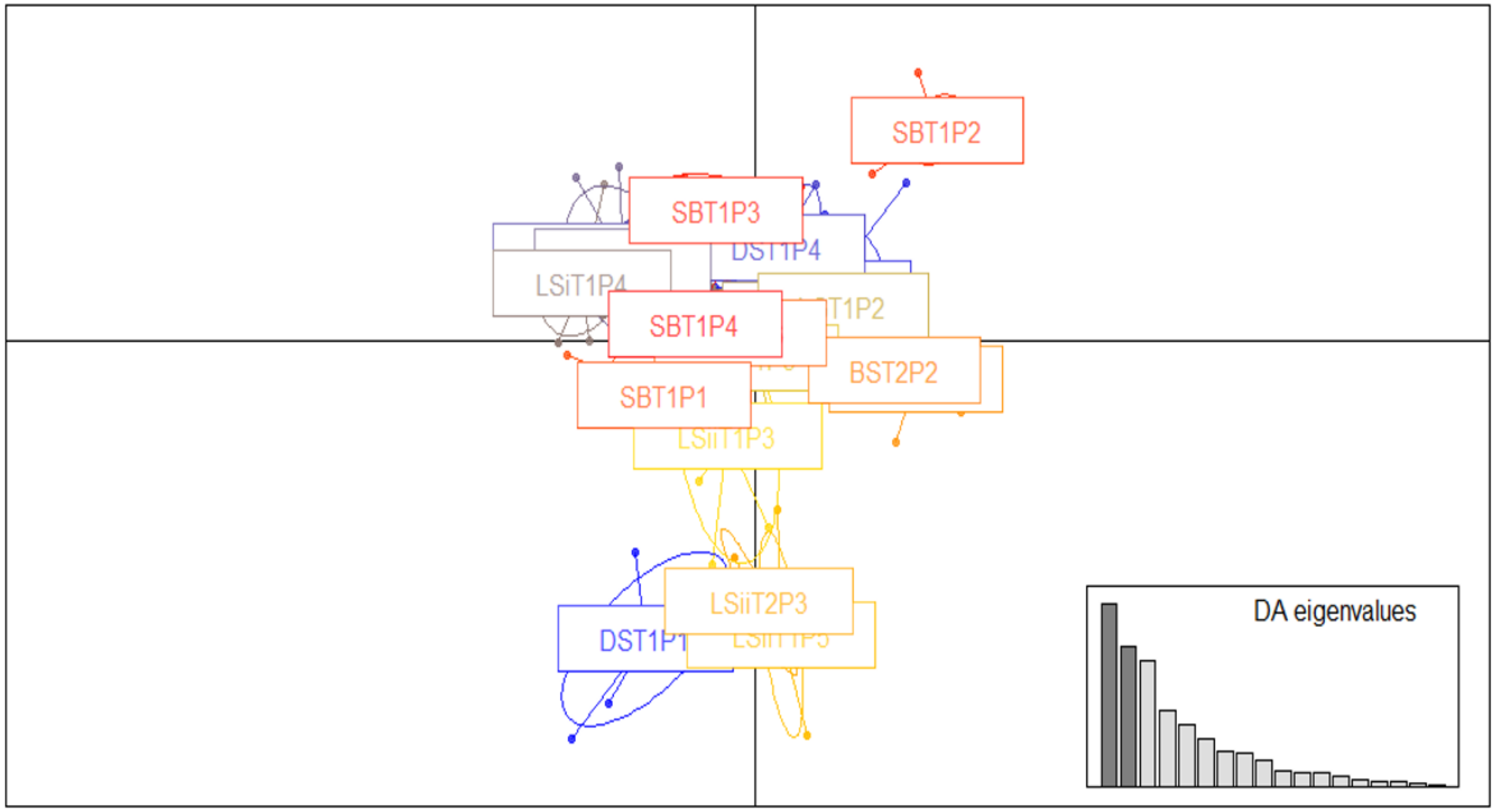

Figure S5.5. Scatterplot from discriminant analysis of principal components (DAPC) of the first two principal components discriminating *Ganoderma philippii* populations by plots (plot location given as *a priori*). Points represent individual observations. Lines and shapes represent population membership. Inertia ellipses represent an analogue of a 67% confidence interval based on a bivariate normal distribution.

Deras plot 4, rotation 1

1	20	21	40	41	60	61	80	81	100
2	19	22	39	42	59	62	79	82	99
3	18	23	38	43	58	63	78	83	98
4	17	24	37	44	57	64	77	84	97
5	16	25	36	45	56	65	76	85	96
6	15	26	35	46	55	66	75	86	95
7	14	27	34	47	54	67	74	87	94
8	13	28	33	48	53	68	73	88	93
9	12	29	32	49	52	69	72	89	92
10	11	30	31	50	51	70	71	90	91

MLLs sampled only once

2 unique MLLs isolated from 1 tree

Langgam plot 3, rotation 2

1	20	21	40	41	60	61	80	81	100
2	19	22	39	42	59	62	79	82	99
3	18	23	38	43	58	63	78	83	98
4	17	24	37	44	57	64	77	84	97
5	16	25	36	45	56	65	76	85	96
6	15	26	35	46	55	66	75	86	95
7	14	27	34	47	54	67	74	87	94
8	13	28	33	48	53	68	73	88	93
9	12	29	32	49	52	69	72	89	92
10	11	30	31	50	51	70	71	90	91

MLL 164

MLL 26

Sebulu plot 2, rotation 3

1	20	21	40	41	60	61	80	81	100
2	19	22	39	42	59	62	79	82	99
3	18	23	38	43	58	63	78	83	98
4	17	24	37	44	57	64	77	84	97
5	16	25	36	45	56	65	76	85	96
6	15	26	35	46	55	66	75	86	95
7	14	27	34	47	54	67	74	87	94
8	13	28	33	48	53	68	73	88	93
9	12	29	32	49	52	69	72	89	92
10	11	30	31	50	51	70	71	90	91

MLL 14

MLL 16

Logas South plot 3, rotation 1

1	20	21	40	41	60	61	80	81	100
2	19	22	39	42	59	62	79	82	99
3	18	23	38	43	58	63	78	83	98
4	17	24	37	44	57	64	77	84	97
5	16	25	36	45	56	65	76	85	96
6	15	26	35	46	55	66	75	86	95
7	14	27	34	47	54	67	74	87	94
8	13	28	33	48	53	68	73	88	93
9	12	29	32	49	52	69	72	89	92
10	11	30	31	50	51	70	71	90	91

MLLs sampled only once

2 unique MLLs isolated from 1 tree

Logas South 2 plot 3, rotation 2

1	20	21	40	41	60	61	80	81	100
2	19	22	39	42	59	62	79	82	99
3	18	23	38	43	58	63	78	83	98
4	17	24	37	44	57	64	77	84	97
5	16	25	36	45	56	65	76	85	96
6	15	26	35	46	55	66	75	86	95
7	14	27	34	47	54	67	74	87	94
8	13	28	33	48	53	68	73	88	93
9	12	29	32	49	52	69	72	89	92
10	11	30	31	50	51	70	71	90	91

MLL 162

MLL 178

Baserah plot 1, rotation 3

1	20	21	40	41	60	61	80	81	100
2	19	22	39	42	59	62	79	82	99
3	18	23	38	43	58	63	78	83	98
4	17	24	37	44	57	64	77	84	97
5	16	25	36	45	56	65	76	85	96
6	15	26	35	46	55	66	75	86	95
7	14	27	34	47	54	67	74	87	94
8	13	28	33	48	53	68	73	88	93
9	12	29	32	49	52	69	72	89	92
10	11	30	31	50	51	70	71	90	91

MLL 92

MLL 156

MLL63

Figure S5.6. Schematic of the spatial arrangement of MLLs in one representative plot from each site. MLLs from individual isolates are in grey, MLLs sampled from more than one tree are in colour. Each number from 1 to 100 represents a tree at planting time though some may have been dead or missing at the time of sampling. Roots of all trees were inspected and isolations attempted from all infected trees, though not all attempts were successful.

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS

6.1. Rationale of my research

Species of *Ganoderma*, particularly *G. philippii*, *G. australe* and *G. mastoporum*, are commonly found in Indonesian *A. mangium* plantations (Farr and Rossman 2012; Lee 2004; Mohammed *et al.* 2014). *Ganoderma philippii* is a root rot pathogen while the other two species are secondary root invaders and wood rotters (Page *et al.* 2018). *G. philippii* is a particularly aggressive pathogen to *A. mangium*, a hardwood plantation species grown on short 5-6-year rotations for pulpwood in Indonesia. The disease becomes progressively worse over each rotation and control measures have met with limited success (Francis *et al.* 2014). By the mid-2000s it was acknowledged that by the third rotation, several sites were found to be no longer capable of providing a commercial yield at harvest (Irianto *et al.* 2006). Growth rates of *A. mangium* plantations in Sumatra in the early rotations ranged between 22 and 35 m3/ha/yr (Harwood 2014). This productivity was then dramatically reduced being impacted by the combination of two fungal diseases (*Ganoderma* and *Ceratocystis*), which reduced growth on infected sites to 15 m3/ha/yr or lower (Nambiar *et al.* 2015). Although a preferred species because of its potential growth rate and pulp quality, *A. mangium* has been largely replaced by *Eucalyptus* (Nambiar *et al.* 2018). In total, by 2016–2017, more than 600 000 ha of the area previously under *A. mangium* had been replaced, much of this by eucalypts (Nambiar *et al.* 2018). The rates and scale of this change driven by diseases losses are unprecedented and associated with unknown consequences for the economy and ecosystems in Indonesia (ITTO 2017). Unfortunately, eucalypts are fast becoming susceptible to the same or similar fungal diseases as *A. mangium* including *G. philippii* (Heru Indrayadi, personal communication).

Root diseases caused by basidiomycetes are among the most studied diseases of trees in the world, for the genera *Armillaria* and *Heterobasidion*, and there are many potential approaches

to their management as summarised by Mohammed *et al.* (2014). Robert Hartig, in 1874, first recommended removal of infested root systems from soil as a means of reducing future infections (Hartig 1874). Stump and root removal, although undoubtedly effective, is, however, often limited to high-value sites, amenable slope and soil type, and certain silvicultural systems, because of cost and potential negative impacts such as soil degradation. An organism within a tree, stump or soil is less easily targeted by chemicals even if these are systemic. Even if partially effective, chemicals are costly, especially at a plantation scale and will usually impact the environment negatively. Their use may only delay infection and pathogen spread. The use of alternative or more disease tolerant hosts is not always straightforward as basidiomycete pathogens are generalist and have wide host ranges; susceptibility may be influenced by many factors that affect both host and pathogen including soil type and host age. Host species that are tolerant to root-rot disease may not always be commercially acceptable. Biocontrol of *H. annosum s.l.* by application of a fungal competitor to stump surfaces to prevent pathogen spore colonization is the only true success story for this approach in the management of a basidiomycete root-rot disease. Current research into managing *Ganoderma* is focusing on biocontrol approaches. Isolations from *Ganoderma* infected roots or colonized debris in Indonesian acacia plantations have given rise to a suite of other isolates being tested for their potential as BCAs (Mohammed et al. 2014). Several of these are showing promise and have been confirmed as belonging to species of *Cerrena* and *Phlebiopsis*. The Indonesian *Phlebiopsis* isolates which, like their European counterparts that combat *H. annosum s.l.*, form oidia, have shown significant antagonistic behaviour against *G. philippii* in laboratory trials but are less easy to successfully apply in the field due to the predominance of manual harvesting and timely accessibility to stumps. Biocontrol is a strategy more likely to be deployed with success over the many thousands of hectares involved in tropical plantations, especially in South-East Asia where topography and climate often prohibit other management approaches such as stump removal and chemical control.

The ability to trade-off concepts such as disease risk with the cost, effectiveness and deployment of various management strategies will depend on how well the pathosystem is understood and the context imposed by the cropping system and commercial environment. For the well-established oil palm and rubber industries in Indonesia, there is already considerable understanding of basidiomycete stem- and root-rot diseases and potential management strategies, even though the literature is not always mainstream and available external to commercial investors. The plantation pulpwood industry based on acacias and eucalypts in the tropics is relatively new compared to oil palm and rubber and has more to learn about the interactions between recently imported non-native host species, basidiomycete root-rot pathogens and their environment. Much can be inferred for *G. phillipii* but very little has been substantiated. The main objective of the present study was to define fundamental aspects of *G. philippii* disease biology and aetiology (such as sexuality, gene flow and capacity for genetic variation, population dynamics) in order to ascertain the importance of basidiospores in disease dispersal and incidence and apply this new knowledge to the management of the disease. The thesis comprised three experimental investigations of; (1) *G. philippii* basidiospore germination, (2) *G. philippii* breeding systems, and (3) population structures of *G. philippii* in acacia and eucalypt plantations. The two saprophytic *Ganoderma* species, *G. australe* and *G. mastoporum* were included in the first two studies, for comparison with *G. philippii*.

6.2. Significance and impact of germination studies

The pioneer study carried out was to describe simple, reliable protocols for *in-vitro* germination of *G. philippii* basidiospores and describe a standardised method for optimum axenic spore germination for *G. philippii, G. australe* and *G. mastoporum*.

Spore germination is a preliminary stage to fungal penetration into the host or colonisation of a woody substrate or recently cut stump and is the mechanism that converts the spore from a dormant biological organism to one that grows vegetatively (Adaskaveg and Gilbertson 1986;

Bazzalo and Wright 1982; Brown and Merrill 1973). The right environmental temperature, available water or moisture and, sometimes, the presence of nutrients transferred from the host or substrate into the water, are the most important environmental factors that break dormancy and aid spore germination (Griffin 1994; Ho and Nawawi 1986b; Page *et al.* 2017).

We modelled the germination dynamics of basidiospores in relation to factors such as spore density, simple or complex carbohydrate availability in the nutrient media, biotic carbon sources such as sawdust and/or ethanol media additives, and incubation temperature. Spore density was also critical to achieving the best germination rate, with ~400 spores/cm^2 optimal for all three species. The best medium for *G. australe* and *G. mastoporum* basidiospore germination was rice dextrose agar with a mixture of *Eucalyptus* and *Acacia* sawdust, whereas for *G. philippii* it was 1% malt extract agar plus ethanol, with or without sawdust. The pathogen *G. philippii* (which does not appear to sporulate as profusely as the two saprophytic species) was shown to have more specific requirements for germination. *Ganoderma australe* and *G. mastoporum* basidiospores germinated on all media, whereas *G. philippii* basidiospores required media that contained 2% ethanol.

The most important aspect to this fundamental scientific investigation is that it successfully demonstrated a simple method for the collection and germination of *G. australe*, *G. mastoporum* and *G. philippii* basidiospores. As discussed, the most promising means of management appears to lie in biological control by competitive wood degrading fungi (Mohammed *et al.* 2014). This germination study paved the way for studies into competition between *G. philippii* basidiospores and oidia of potential biological control agents on stumps of felled trees, such as have been conducted in other pathosystems (Oliva *et al.* 2015; Sun *et al.* 2009). The availability of a simple method for axenic spore germination was required to obtain monokaryons and study the mating systems of *Ganoderma* species found in Indonesian

acacia plantations. Monokaryon isolates facilitated my study of the population genetics of G. *philippii* and the development of molecular markers.

6.3. Significance and impact of investigating the sexual biology of *G. philippii, G. mastoporum,* and *G. australe*

This investigation was undertaken to determine the sexuality and mating systems of *G. philippii* and co-occurring *Ganoderma* species, observing the somatic interactions between monokaryotic and dikaryotic mycelia and noting any incompatibility mechanisms. Ten monokaryotic siblings harvested as single spore isolates from each of two *G. philippii*, one *G. mastoporum* and two *G. australe* basidiocarps were paired in every possible combination. In all three species monokaryons were self-sterile. By examining the contact-zone hyphae, it was determined that in all three species, full sexually compatible matings occurred in 26–33% of the crossings. Two mating type loci were identified. Dikaryons generated from monokaryotic isolates showed morphological changes as cultures aged.

These results were not unexpected but important to confirm. Almost 60% of the higher basidiomycetes are characterised by a more complex bifactorial or tetrapolar incompatibility system, involving two mating types with many alleles i.e. mating competence is determined and two individuals will only be compatible if they have different specifications for each factor A and B. The genetics of sexuality for several G*anoderma* spp. have been determined previously and the bifactorial system predominates within the genus (Adaskaveg and Gilbertson 1986; Banerjee and Sarkar 1958; De 1980; Triratana and Chaiprasert 1991). The results of this study indicated that outcrossing is favoured in all three species, *G. australe*, *G. philippii* and *G. mastoporum* which is a system that promotes adaptation to new environments (Rajchenberg 2011).

6.4. Significance and impact of *G. philippii* population genetics study

The incidence and severity of red root disease increases with rotation. This may be linked to changes in the mode of dispersal of G. *philippii,* the balance between vegetative and spore dissemination. Root to root spread has previously been implicated in the spread of *Ganoderma* root rot in *Acacia* plantations in Indonesia, based on spatial analysis (Francis *et al.* 2014) and is a common strategy of many basidiomycete root rot pathogens (Anderson and Kohn 1995; Ariffin *et al.* 1996; Chase and Ullrich 1983; Dart *et al.* 2007; Flood *et al.* 2000b). Early efforts to manage this disease were therefore based on sanitation and the removal of stumps. This approach in extensive plantations was found to be cost-prohibitive and not significantly effective (Rimbawanto *et al.* 2009). Preliminary SI tests indicated that genetic diversity increased with successive rotations and an increasing, not decreasing role of spores, an observation which is counter-intuitive to the vegetative spread concept (Mohammed *et al.* 2014). In order to explain why stump removal was not an effective disease reduction strategy and to better inform future management strategies (e.g. the targeted application of biocontrol agents) we studied the population genetics of *G. philippii.* Our aim was to evaluate the role of sexual and asexual reproduction in its mode of spread with microsatellite analysis.

Primers to enable microsatellite analyses were screened against three isolates of *G. philippii* from geographically separate locations. All but two of the primer pairs successfully amplified *G. philippii* DNA, but the majority amplified a single product that did not vary among the test isolates. Eight of the primer sets were polymorphic and heterozygous in at least one of the test isolates. Analysis of monosporic isolates indicated whether the microsatellite markers used segregate independently and therefore fewer markers are required to achieve the same P values in my population study.

Genetic diversity remained high at all sampling scales, irrespective of rotation, plot, site or region. Nevertheless, it is unclear whether populations in second and third rotation plantations

establish mainly from persistence of woody inoculum or establishment of new infections from basidiospores. While the greatest genetic variation was detected between populations (sites), genetic distance is not correlated with geographic distance. This may be explained by largely in-breeding populations with occasional immigrants from further afield. This explanation is supported by the results which show clusters of very closely related individuals at each site with scattered individuals from one location that are more closely linked genetically to a different population. The Sebulu population, which is on a different island, is no more genetically distinct from the Sumatran populations than they are from each other.

It appears that the best description of the population structure of *G. philippii* is one resulting from mixed modes of reproduction or mating that include clonality (vegetative spread) sexual recombination between siblings as well as outcrossing. Although *G. philippii* has a tetra polar mating system which favours out-crossing (Page *et al.* 2018) other factors which increase the likelihood of sibling mating may predominate. These factors could be the location of *G. philippii* sporocarps low on the trunk so it is more difficult for spores to become airborne. Basidiospores of *G. philippii* are also produced in relatively lower abundance and are more fastidious in their requirements for germination compared to *G. australe* or *G. mastoporum* (Lim 1977; Page *et al.* 2017). However basidiospores travel a long distance (our evidence suggests between island travel is possible) but it could be human or fly assisted e.g. excreted by tipulid flies in the genus *Limonia* (as G. pseudoferreum Lim 1977).

Knowledge of a pathogen's mode of reproduction has implications for disease management, as shown by a related pathogen, *G. boninense,* which causes basal stem rot in oil palms in Malaysia and PNG. Basidiospores have been implicated as the main inoculum source for *G. boninense* (Mercière *et al.* 2017; Pilotti *et al.* 2003) and infection mechanisms for basidiospores have been elucidated (Rees *et al.* 2011). Studies based on mitochondrial DNA markers, mating alleles, somatic (or vegetative) compatibility and microsatellites have all

revealed high levels of genetic diversity (Mercière *et al.* 2017; Pilotti *et al.* 2003). However the population structure of *G. boninense* contrasts strongly with that of *G. philippii,* showing little differentiation among populations across northern Sumatra and Peninsular Malaysia (Mercière *et al.* 2017) and apparently comprised of one large, freely recombining population, with little clonal duplication. Understanding the biology of *G. boninense* at these levels has informed changes in disease management (Mercière *et al.* 2017): early management strategies focused on the removal of stump and debris inoculum but now focus on the removal of dead fronds, the bases of which can be the location of new infections (Rees *et al.* 2011).

A similar strategy to the treatment of harvested stumps with *Phlebiopsis gigantea* to prevent the germination of spore of *Heterobasidion annosum* (Pratt 1998) has been proposed for the *G. philippii/A. mangium* pathosystem (Agustini *et al.* 2014b). This may be an effective strategy to prevent new infections in disease-free areas and indeed the treatments of stumps in new areas in the early 2000s with urea and lime did prevent infection (Heru Indrayadi, personal communication). Researchers were puzzled when clear reduction in early experiments were not repeatable at other sites but given the propensity for clonal spread, is not expected to eradicate the pathogen from plantations if inoculum was pre-existing. As mechanical stump removal is relatively expensive and unlikely to remove all infected roots, additional strategies to manage woody inoculum are required. Current strategies focus on the inoculation of nursery seedlings with biocontrol agents that will survive in the rhizosphere after planting or persist endophytically (Hill *et al.* 2010; Mohammed *et al.* 2014; Prasad and Naik 2002; Sariah 2003; Sariah and Zakaria 2000).

6.5. The urgent need for effective disease management in hardwood plantations

The Indonesian government is eager to expand production capacity of the national pulp and paper industry because the success of Indonesia's pulp and paper industry comes on the back of ever-increasing global demand for paper (Setyawati 2017). The current target is to raise

capacity from 7.93 million tons per year to 10.53 million tons. According to a recent industry statement, the pulp and paper industry accounts for 6.7% of Indonesia's gross domestic product generated by components of processing industries, employs 260 000 workers directly and 1.1 million workers indirectly, and in 2016, it ranked as the country's seventh largest foreign exchange earner contributing US$3.8 billion (FAO 2016). In addition to the pulp and paper companies, plantations are also owned and managed by many small to medium wood growing enterprises (Ministry of Forestry 2007; Mohammed *et al.* 2012). The large pulp and paper companies also support rural communities in tree growing whether these are forest or horticultural trees. The economic and social impacts of the forest sector in Indonesia are important (ITS Global 2011). In some regions such as Riau in Sumatra the net impacts of the sector on economy, tax revenue, employment (with high job multiplier effects) and household income are substantial and critical (Nambiar *et al.* 2018). Policy makers and investors need clear information about the impacts of industrial timber plantations to better design and manage plantations and to facilitate their integration into the rural landscape to maximise socio-economic impact. The serious impact of pests and pathogens in Indonesia's forest plantations obscures clarity of information making forest health and biosecurity top priorities for research and development (Nambiar *et al.* 2018).

6.7. Research directions

The research in this thesis can be used as a platform for fundamental scientific studies (e.g. of the ecology of *Ganoderma* species) to more applied research of immediate application to disease management i.e. investigations of:

- the ecology and dispersal mechanisms of the three species studies in this thesis and their ecological relationships. *Ganoderma philippi* in acacia is difficult to isolate from old infection centres and *G. australe* and *G. mastoporum* are more frequently isolated from such centers (personal observation)

- isolates to demonstrate if there is any indication of adaptation to a host or is the gene flow between different host species unrestricted
- the level of variation among *Ganoderma* isolates and the relationship of this variation to pathogenicity or tree mortality
- spore populations (caught in traps) to examine spore dispersal both spatially and temporally
- the mechanisms of dispersal such as whether the passage through an insect is beneficial as suggested by Lim 1977 (although the role of spore dispersal mutualism remains equivocal in many fungus-insect assemblages and has been disproved with another *Ganoderma* species) (Kadowaki *et al.* 2011) and that insects play a role in spore dispersal
- the degree to which *G. philippi* can compete saprophytically or is a "successful" saprophyte and the length of time this pathogen and soil depth at which this pathogen survives in stumps or roots left undisturbed
- if biocontrol agents (endophytes) developed for targeting *Ceratocystis* acacia wilt also reduce the impact of *Ganoderma* root rot

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